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Neuromodulation by endogenous Interlukin-1β in hippocampus of the murine brain: Regulation of neuronal excitation

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ABSTRACT

The pro-inflammatory cytokine, Interleukin-1β (IL-1β), is well known for its ability to initiate and propagate inflammatory responses at sites of infection and tissue injury. Paradoxically at odds with this classic view, it is now clear that IL-1β signaling modulates a number of physiological functions in the central nervous system. In this regard, IL-1β is involved in sleep and body fluid regulation in the basal forebrain and hypothalamus, respectively, and plasticity changes that underlie cognition in the hippocampus. Evidence from a previous study in my laboratory further suggests that IL-1β regulates the innate seizure threshold, which arguably is a reflection of the homeostatic balance between excitation and inhibition (E/I) in the brain. Recognizing that the IL-1β signaling receptor, Interleukin 1 Receptor 1 (IL-1R1), is highly concentrated on granule neurons of the dentate gyrus of the hippocampal formation and that dysfunction of this brain region can shift the E/I balance toward excitation, my research focused on the possibility that constitutive IL-1β signaling in the hippocampal formation modulates brain E/I balance. The specific goal was to elucidate the cellular source(s) and release mechanism of IL-1β in the hippocampus and to examine the functional significance of this release in maintenance of the seizure threshold. In Aim 1, I used the PTZ acute seizure model and mice lacking IL-1R1 to confirm the previous results demonstrating that IL-1β signaling is required for maintenance of the innate seizure threshold. In Aim 2, using a brain-permeable antagonist of the ATP-activated purinergic receptor, P2RX7, I found that IL-1β immunoreactivity accumulated in pyramidal neurons of the CA3 and to a lesser extent in the CA1 subregions of the hippocampus. A subsequent study using hippocampal neuron cultures indicated P2RX7-dependent neuronal release of IL-1β and found that its subcellular localization included both cell bodies and processes of these neurons. Thirdly, I found that P2RX7 increased

activity-dependent gene expression in cultures of hippocampal neurons and lowered the seizure threshold in a manner that resembled the phenotype of mice lacking IL-1R1 signaling. Together, results from this aim indicated strong possibility of basal IL-1β release from hippocampal neurons occurring via an ATP-dependent mechanism and contributing to maintaining the E/I balance of the normal brain. Additional results from studies in Aim 3 suggest that the rate of production and release of IL-1β is not affected by changes in neuronal excitation and that IL-1β may affect neuronal excitation via modulation of cyclooxygenase-2 function in hippocampus. Overall, the results from my dissertation research extend the knowledge of the biological function of IL-1β in the normal brain. A better understanding of this function could facilitate development of novel therapies to treat seizure induction in epileptic brains and perhaps reduce the probability of acquiring epilepsy in at-risk individuals.

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LIST OF ABBREVIATIONS:

ATP	Adenosine triphosphate
APV	Amino-5-phosphonovaleric acid, competitive NMDA antagonist
2-ME	β-mercaptoethanol
AA	Arachidonic acid
AAALAC	The Association for Assessment and Accreditation of Laboratory Animal Care International
b.w.	Body weight
BSA	Bovine Serum Albumin
CA1	Cornu Ammonis 1
CA3	Cornu Ammonis 3
cDNA	complimentary DNA
COX	Cyclooxygenase
CTCF	Corrected total cell fluorescence
DAPI	Diamidino-2-phenylindole, nuclear dye
DG	Dentate Gyrus
DMSO	Dimethyl sulfoxide, solvent
DNA	Deoxyribonucleic acid
E/I	Excitation-inhibition
EDTA	Ethylenediaminetetraacetic acid
EPSC	Excitatory post synaptic current
FIJI	FIJI is just ImageJ, a software for multimodal photo analysis
GABA	γ-aminobutyric acid
HS	Horse serum
i.p.	intraperitoneal, mode of injection
IACUC	Institutional animal care and use committee
ICC	Immunocytochemistry
ICE	Interleukin-1β converting enzyme, otherwise Caspase-1
IHC	Immunohistochemistry
IL-1β	Interleukin-1β
IL-1R1	Interleukin-1 receptor 1
IL-1RacP	Interleukin-1 receptor associated protein
IRAK	Interleukin-1 receptor associated kinase
JNJ	JNJ-47965567, brain permeable P2X7R receptor antagonist
kb	Kilo-base pair, 1000 DNA base pairs
kDa	Kilo-Dalton, used for measuring molecular weight of peptides
KO	Knock out
LTP	Long term potentiation
MAP-2	Microtubule-associated protein 2

MK-801	Dizocilpine, non-competitive NMDA antagonist
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response 88
NIH	National Institute of Health
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain
NMDA	N-methyl-D-aspartate
NREM	Non-rapid eye movement
P2X7R	Purinergic 2 X(ionotropic) receptor 7
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PGES	PGE_2 synthase
p.o.	Oral gavage
Ptgs2	Prostaglandin endoperoxide synthase 2, otherwise Cox-2
PTZ	Pentylenetetrazol
REM	Rapid eye movement
RNA	Ribonucleic acid
s.c.	Sub-cutaneous, mode of injection
SBE-β-CD	Sulphobutyl ether-b-cyclodextrin, lipophilic solvent
TRAF-6	TNFα receptor associated factor 6
v/v	Volume per volume
w/v	Weight by volume
WT	Wild type
Ac-yVAD-CHO	Caspase-1 inhibitor
PDL	Poly-*D*-Lysine
PLL	Poly-*L*-Lysine
Ara-C	Cytosine arabinoside

Chapter 1: Background

1.1 Overview

Seizure, the hallmark symptom of epilepsy on a physiological level occurs due to excessive neuronal activity that happens due to the lowering or imbalance of an innate brain property, seizure threshold (physiological synchrony of excitatory and inhibitory neurotransmitter system for maintenance of brain homeostasis). Several endogenous neuromodulators have been found to play a role in the maintenance of this physiological balance. One such neuromodulator is the cytokine, Interleukin-1β. Interleukin-1β is shown to regulate the maintenance of seizure threshold, and this dissertation shall further characterize the biology of its regulatory role in the hippocampus of the limbic cortex within central nervous system utilizing a murine system of acute seizure and hippocampal neuronal cultures.

1.2 Epilepsy

Epilepsy is a clinical term that has been defined at http://www.ilae.org/ as "a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain" (Fisher et al. 2014). Epilepsy is a chronic brain disease associated with cognitive, psychological, and learning deficits alongside other pathophysiological and social sequelae (Fisher et al. 2014; Bialer et al. 2006).

Epilepsy is one of the most prevalent neurological diseases affecting every aspect of demographics and can occur alone or combined with other neurological conditions such as autism, Alzheimer's disease, brain injury or brain cancer (Gaitatzis, Trimble, and Sander 2004; Pitkänen et al. 2014; Bozzi, Provenzano, and Casarosa 2018). Epilepsy affects over 50 million people worldwide, particularly people under the age of 20 or above 60 years (de Boer, Mula, and Sander 2008; Bialer et al. 2006). It has been estimated that there is a 1 in 26 people have risk of acquiring epilepsy in their lifetime (Sirven 2015). Pharmacological or neuromodulatory (e.g.

deep brain stimulation) intervention have been developed that are effective in suppressing the symptoms of epilepsy but not correct it. However, some patient populations may develop resistance or may be unresponsive to the pharmacokinetics, making this condition harder to manage clinically (Beghi et al. 2006).

The neurobiology of epilepsy is complicated, a number of risk factors increases the likelihood of acquiring epilepsy through the process of epileptogenesis. These risk factors, such as brain trauma, ultimately lead to cascade of molecular and cellular events causing permanent shift towards hyperexcitable state (Scharfman 2007; Staley 2015). Every cell types of brain including endothelium and glia in addition to neurons can be involved in this process (Avoli et al. 2005). Over last few decades, studies in humans have refined the diagnostic criteria and studies from models have helped in better understanding the molecular mechanisms that underlies the disease progression and neurobiological alterations in the epileptic brain (Bradford 1995; Fisher 1989; Scharfman 2007; De Lanerolle, Lee, and Spencer 2010). While this has contributed to development of better anti-epileptic therapies, much remains unknown about the pathogenesis of epilepsy.

1.2.1 Classification of epilepsy

The International League Against Epilepsy (ILAE) has sought to classify epilepsy to more clearly define this complex disease. Initially, ILAE classifies diagnostic scheme of epilepsy based on ictal phenomology, seizure types, syndrome, etiology and impairment (Engel 2006). Subsequently, these classifications have been revised and updated to better understand this disease progression and cure (Engel et al. 2011; Fisher et al. 2014; Pack 2019).

The International League Against Epilepsy classifies epilepsy on three levels- the epilepsy type, the seizure type, and the epilepsy syndrome (https://www.epilepsydiagnosis.org/epilepsy/

epilepsy-classification-groupoverview.html). Epilepsy type is classified based on abnormal electrical activity: generalized (involving whole brain at the onset), focal (discrete brain region such as temporal lobe), combined general and focal and of unknown origin. The syndrome on the other hand is classified based on the age of the patient when epilepsy occurs. Epileptic seizure types primarily are self-limiting seizures which includes absence (formerly petite mal) or convulsive (formerly grand mal) seizure and continuous seizures which includes generalized and focal *status epilepticus* (convulsive or nonconvulsive seizure which lasts for longer than 5 minutes or when seizures occur close together and the individual doesn't recover in between seizures) (Engel 2006; Shorvon 2011).

Epilepsy is also classified on the basis of etiology, they are idiopathic, symptomatic, provoked and cryptogenic (Shorvon 2011) (https://www.epilepsydiagnosis.org/aetiology /epilepsies-etiology-groupoverview.html). For such a complex disease, it is obvious, one classification of the disease is not sufficient. Hence the multi-aspect classification of the disease helps provides a better understanding of the disease for the clinicians, researchers, and patients.

Various aspects of epilepsy have been reviewed over years (Fisher 1989; Sloviter 2005; Pitkänen and Lukasiuk 2009; Vezzani et al. 2013; Scharfman 2007; Staley 2015). Previous students from my research lab have reviewed epilepsy in their theses. Claycomb (Claycomb 2011) provided comprehensive review on neurobiology of epilepsy and Gong (Gong 2018) included a review of the genetic basis of epilepsy.

For my dissertation study, I will focus on the mechanism that underlies the homeostatic balance of excitatory and inhibitory functions of CNS, in particular to the role of Interleukin-1β in the maintenance of that homeostatic balance. To study homeostatic balance of excitatory and inhibitory functions, I will utilize acute seizure model, which causes a shift in this balance to

favor excitation. An epileptic seizure is the key symptom of epilepsy, which is an abnormal synchronous electrical activity of populations of neurons in the brain (Bialer et al. 2006; Fisher et al. 2014).

1.2.2 Seizure and seizure threshold

Seizures are key symptoms of epilepsy. Seizures are consequences of abnormal synchronous electrical activity in the populations of neuron in the brain (Bialer and White 2010; Fisher et al. 2014). A seizure however does not necessarily mean the person would have epilepsy. Epilepsy is defined as occurrence of two or more seizure independent of each other (Engel 2006; Fisher et al. 2014). The seizure threshold is the innate biological property of the brain that is defined by "a concept that seeks to reconcile the balance of inhibitory and excitatory neuronal stimuli with an individual predisposition to seizures" (Wills, Theeler, and Ney 2009; Oh and Bainbridge 2012; Bozzi, Provenzano, and Casarosa 2018). Therefore, a seizure is an aberrant occurrence that happens either due to excessive excitatory neuronal activity or absence or failure of inhibition of neuronal excitation or both (Wills, Theeler, and Ney 2009; Fisher et al. 2014).

1.2.3 Excitation/inhibition balance in CNS

In CNS, neuronal activity is essentially a balance or homeostasis between excitation and inhibition [glutaminergic and GABAergic functions (Cline 2005; Žiburkus, Cressman, and Schiff 2013)]. Neuronal activity is a physiological process in the central nervous system (CNS) which maintains homeostatic balance of excitatory and inhibitory (E/I) functions to create effective synapse (anatomical zone for functional communication between neurons). Neuronal communication over time becomes effective by developing synaptic strength (homeostatic synaptic plasticity). Synaptic plasticity is important for sustaining several physiological functions of the brain (Žiburkus, Cressman, and Schiff 2013; He and Cline 2019), including

memory formation and adult neurogenesis (Saaltink and Vreugdenhil 2014; Lopatina et al. 2019). Billions of neuronal connections utilize intrinsic homeostatic modulation (synaptic scaling) to achieve synaptic plasticity which in turn conserves the homeostatic excitatory and inhibitory mechanisms in CNS (Turrigiano 2012).Any deviation in the intrinsic homeostatic modulations which keeps the E/I balance in place can trigger neuronal disorders (Lopatina et al. 2019). To site example, homeostatic excitation/inhibition balance is altered due to neurodevelopmental disorders or environmental or metabolic stress (Saaltink and Vreugdenhil 2014).

Deviation in the homeostatic excitation/inhibition balance can trigger neuronal dysfunction as exemplified by seizure and epilepsy. Bonansco et al. comprehensively review how glutamatergic (excitatory) plasticity and GABAergic (inhibitory) plasticity in epileptic brain differs from the normal brain (Bonansco and Fuenzalida 2016). Similarly, a hypothesis behind autism spectrum disorder-epilepsy co-morbidity is a multi-etiological factor of neurodevelopment (genetic, metabolic, environmental) leading to an altered state of excitatory and inhibitory structure resulting in persistent excitation/inhibition imbalance (altered seizure threshold) and neuronal hyperexcitability (seizure) (Bozzi, Provenzano, and Casarosa 2018). Likewise, decreased inhibition leading to unchecked oscillatory activity leads to seizure (Bozzi, Provenzano, and Casarosa 2018) or imbalance in striatal-thalamocortical connection causes motor and cognitive impairment leading to Parkinson's disease (Llinás et al. 1999) or reduced GABAergic signaling presented in autism (Nelson and Valakh 2015).

Seizure threshold, therefore, can be reiterated as balance between excitatory (glutaminergic) and inhibitory (GABAergic) forces in the brain which affects the susceptibility of an individual

to seizures and seizure can henceforth be described as altered excitation/inhibition balance where excitation is favored.

1.2.4 Model to study alteration in E/I balance

Alterations in E/I balance can be studied either through electrophysiology (recording of electrical activity) (Žiburkus, Cressman, and Schiff 2013) or via perturbing the E/I balance by inducing hyperexcitability via certain chemical agents (Steppuhn and Turski 1993), sounds (Wieraszko and Seyfried 1989) or electric stimuli (Zatz and Roth 1975), etc. In my dissertation research, I employed the chemo-convulsant, pentylenetetrazol (PTZ) as a model to perturb the E/I balance in mice and bicuculline in cultured hippocampal neurons in order to understand physiological mechanism that maintains it.

Pentylenetetrazol (PTZ): Pentylenetetrazol or PTZ is a GABA receptor inhibitor (MacDonald and Barker 1978) that binds to the picrotoxin binding site of $GABA_A$ receptor (Ramanjaneyulu and Ticku 1984). PTZ has been used extensively to test the efficacy of anti-seizure drugs in response [reviewed in (Krall et al. 1978)]. It is also broadly used to study acute seizure activity in laboratory animals (Steppuhn and Turski 1993). In this regard, it is used as a model of neuronal hyperexcitability to study the molecular mechanisms that maintains the E/I balance (seizure threshold) in normal brain. PTZ is also used to gradually sensitize the brain in a model called kindling (Da Silva, Pereira, and Elisabetsky 1998) which is a process of acquiring epilepsy (epileptogenesis).

PTZ induced seizures may be convulsive or non-convulsive depending on the dosage (Claycomb, Hewett, and Hewett 2011). Acute PTZ induced seizures occur rapidly (within minutes) in a progressive fashion. Initially, animals become non-responsive, followed by short period of myoclonic muscle jerks that may transition to convulsive seizure behaviorally

exhibited by tonic-clonic jerks. This progression of seizure activity was classified by Ronald Racine (Racine 1972a; 1972b; Racine, Okujava, and Chipashvili 1972) and revised later (Lüttjohann, Fabene, and van Luijtelaar 2009).

In addition to studying the PTZ stimulated molecular and biochemical changes that occur in CNS (Yount, Ponsalle, and White 1994; Ferraro et al. 1999; Kliouеva et al. 2001), researchers have recorded activation of BOLD-fMRI signals triggered by PTZ during seizure (Keogh et al. 2005; Brevard et al. 2006) to study seizure circuit. PTZ induced acute seizure ultimately resolve within minutes without causing tissue damage or inflammation (Claycomb, Hewett, and Hewett 2011), therefore ascertains to be the optimal model to study E/I balance.

For studies herein, PTZ will be injected intraperitoneally and seizure behavior assessed using a seizure scale modified from Racine scale (Claycomb, Hewett, and Hewett 2011) and explained in methods section 2.3.3 of Chapter 2).

Bicuculline: Bicuculline, first identified in 1970, is a competitive GABAA receptor antagonist, inducing GABA mediated synaptic inhibition (MacDonald and Barker 1978; Steppuhn and Turski 1993). The role and function of bicuculline have been reviewed thoroughly by G. Johnston (Johnston 2013). Bicuculline is utilized as a chemo-convulsant in experimental animals where GABA receptor antagonism by indirect disinhibition shifts the excitation/inhibition balance favoring excitation (Dhir, Naidu, and Kulkarni 2006; Librizzi et al. 2012).It is also used in cultured neurons to shift E/I balance via disinhibition (Stark and Bazan 2011). However, bicuculline is chemically not stable and is therefore administered in the form of quaternary salts (Johnston 2013). For studies herein, disinhibition in cultured hippocampal neurons will be elicited with bicuculline methobromide treatment, quaternary salt of bicuculline and

transcriptional activity of the immediate early genes, c-Fos will be assessed [(Gong 2018) and explained in section 3.3.3.2 Of chapter 3).

1.3 IL-1β: The protein of interest

Several modulatory proteins have been implicated in regulation of the excitatory/inhibitory balance in the brain. Of importance to my research, several cytokines, which have been extensively studied for their role in the immunological system, have been now implicated in contribution of maintenance of the E/I balance of the brain (Vitkovic et al. 2000; Li et al. 2011; Friedman and Dingledine 2011). These cytokines were initially studied for their contribution in the pathobiology of several neurological diseases including seizure and epilepsy. Alterations in the levels of these cytokines in neuroinflammatory or neurodegenerative conditions also cause a shift or tilt in this excitatory/inhibitory balance (Turrin and Rivest 2004).

Of these, IL-1β, TNF-α, IL-6, etc. have been shown to have physiological roles in brain independent of their classical immune functions (Krueger et al. 1998; Vitkovic et al. 2000; Plata-Salamán et al. 2000; Turrin and Rivest 2004; Chennaoui et al. 2015; Yirmiya and Goshen 2011; McAfoose and Baune 2009). The dual functions of these well-known molecules in both physiology and in pathophysiology are, therefore, currently receiving much attention in this regard.

The following section will discuss the biology of one such cytokine – Interleukin 1β (IL-1β) and its role in brain physiology. As a pro-inflammatory cytokine of the immune system, IL-1β is primarily known to be an important mediator, under pathophysiological conditions of the brain, including pathophysiology of seizure and epileptogenesis (Rothwell and Luheshi 2000; Gibson, Rothwell, and Le Feuvre 2004; Simi et al. 2007; Rijkers et al. 2009; Vezzani et al. 2011; Maroso

et al. 2011; Hewett et al. 2012). This section will primarily review the endogenous modulatory role of IL-1β in certain physiological brain functions independent of its classical neuro-immune functionalities.

1.3.1 The Interleukin-1 family

Interleukin 1β belongs to the IL-1 family of Toll-like receptors superfamily. It consists primarily of with seven agonist ligands (IL-1, IL-1β, IL-18, IL-33, IL-36, IL-36β, IL-36), three receptor antagonists (IL-1Ra, IL-36Ra, IL-38) and one anti-inflammatory cytokine (IL-37). These are listed with key specifics in table 1.1 [modified from (van de Veerdonk and Netea 2013; Garlanda, Dinarello, and Mantovani 2013)]. The receptor of IL-1β belongs to IL-1 receptor family (IL-1R) formed from 6 receptor chains, forming four functioning signaling receptor complexes, two decoy receptors, and two negative regulators (Boraschi and Tagliabue 2013). Detailed structure, function and other biological activities of all the members of IL-1 family have been reviewed extensively by several authors (van de Veerdonk and Netea 2013; Garlanda, Dinarello, and Mantovani 2013; Mantovani et al. 2019; Boraschi and Tagliabue 2013; Garlanda et al. 2013).

Cytokine	Alternative names	Receptors	Alternate names	Co-receptor	Alternate names
IL-1 subfamily					
IL-1α	IL-1F1	IL-1R1, IL-1R2		IL-1RacP	IL-1R3
IL-1β	IL-1F2	IL-1R1, IL-1R2		IL-1RacP	
IL-1Ra	IL-1F3	IL-1R1			
IL-33	IL-1F11	ST2	IL-1R4	IL-1RacP	
IL-18 subfamily					
IL-18	IL-1F4	IL-18Rα	IL-1R5	IL-18Rβ	IL-1R7
IL-37	IL-1F7	IL-18Rα			
IL-36 subfamily					
IL-36α	IL-1F6	IL-36R/IL-1RrP2	IL-1R6	IL-1RacP	
IL-36β	IL-1F8	IL-36R/ IL-1RrP2		IL-1RacP	
IL-36	IL-1F9	IL-36R/ IL-1RrP2		IL-1RacP	
IL-36Ra	IL-1F5	IL-36R/ IL-1RrP2			
IL-38	IL-1F10	IL-36R/ IL-1RrP2			

Table 1.1. Nomenclature of members of the IL-1 family with their receptors and co-receptors.

1.3.2 IL-1β Signaling pathway

IL-1β is a secretory protein, signals through the functional plasma membrane receptor complex of Toll-like receptor superfamily (Barton and Medzhitov 2003; Garlanda et al. 2013) consisting of the ligand-binding chain, Interleukin-1 receptor 1 (IL-1R1) (McMahan et al. 1991; Sims et al. 1993). IL-1Receptor accessory Protein (IL-1RacP or IL-1R3) binds to the Toll IL-1 Receptor (TIR) domain of the cytoplasmic tail of the ligand bound IL-1R1 to form a high-affinity receptor complex (Dinarello 2009; Weber, Wasiliew, and Kracht 2010a; Boraschi and Tagliabue 2013). Upon ligand binding, Myeloid differentiation primary response 88 (MyD88) is recruited to this complex. It serves as an adaptor protein for binding and autophosphorylation of serine threonine kinase, Interleukin receptor-associated kinase-4 (IRAK-4). IRAK-4 subsequently phosphorylates the IRAK-1 to recruit and oligomerize Tumor necrosis factor associated factor 6 (TRAF-6) which initiates downstream signaling via p38 Mitogen associated Kinase (MAPK) and Nuclear Factor -κB (NF- κB) (Axel Weber, Wasiliew, and Kracht 2010; Boraschi and Tagliabue 2013; Mantovani et al. 2019). In CNS, IL-1β signaling in neurons only induces p38 MAPK whereas

both p38 MAPK and NF- κB are induced in astrocytes, yielding distinct functional response of IL-1β based on cell types (Srinivasan et al. 2004)

Canonical IL-1β signaling is constrained by a decoy receptor and a naturally occurring antagonist. The decoy receptor of IL-1β, IL-1RII cannot transduce signal owing to its short cytoplasmic tail due to absence of TIR domain (Colotta et al. 1993; McMahan et al. 1991) and thus functions to curb any downstream IL-1β signaling. In addition, a naturally occurring antagonist of IL-1, IL-1receptor antagonist (IL-1ra, Anakinra) inhibits IL-1β signaling by binding to IL-1R1 (Simi, Tsakiri, et al. 2007; Dinarello 2009)**.** Several studies, including studies in CNS, have used exogenous IL-1ra to block IL-1β signaling and perform sensitive assessment of IL-1β function in those specific mechanism (Dinarello 2004).

IL-1RacPb (AcPb), an isoform of IL-1RacP, the accessory protein of IL-1R1, is only present in CNS and constitutively expressed by neurons (Huang et al. 2011). IL-1RacPb mediates Src kinase phosphorylation, which in turn have shown to moderate direct neuronal response such as phosphorylation of ion channel, NMDA induced calcium influx etc.(Viviani et al. 2003; Salter and Kalia 2004; Huang et al. 2011).

1.3.3 Localization in the brain – the ligand and its signaling components

Constitutive expression of IL-1β mRNA was first found in the hypothalamus, hippocampus and cerebellum of rat brain, in the granule cells of the dentate gyrus and in cerebellar Purkinje cells (Yabuuchi et al.1993). As researched and reviewed by multiple researchers, presence of low levels of IL- 1β immunoreactivity is found throughout the brain of rodents in normal conditions, particularly in the hippocampus, hypothalamus, and basal forebrain (Breder, Dinarello, and Saper 1988; Lechan et al. 1990; Watt and Hobbs 2000).

Several cell type in CNS is capable of expressing IL-1β, as being reported in microglia (Giulian et al. 1988), astrocytes (Lieberman et al. 1989), oligodendrocytes (Blasi et al. 1999) and pertinent to this research study, neurons (Lechan et al. 1990; Breder, Dinarello, and Saper 1988; Watt and Hobbs 2000; Kaneko et al. 2006; Gomes et al. 2013; Viviani et al. 2014). These cells are also capable of responding to IL-1β as each cell type expresses fuctional IL-1β receptor, IL-1R1 (Farrar et al. 1987; Cremona et al. 1998; Ban and Milon 1991; Ban 1993; French et al. 1999; Friedman 2001; Hammond et al. 1999; Pinteaux et al. 2002; Nadjar et al. 2005). Recently, a study involving Cre-lox technology was able to place IL-1R1 expression in different cell types of CNS, which includes DG neurons, astrocytes, ventricular and endothelial cells (Liu et al. 2019). Rat hippocampal neurons in culture also express IL-1R1 primarily in the postsynaptic density, alongside MyD88 and IL-1RacP (Viviani et al. 2003). Separate novel isoform of IL-1RacP, IL-1RacPb, that is present in neurons, which mediates an alternate signaling pathway (Smith et al. 2009; Huang et al. 2011). I have shown strong basal expression of IL-1R1 in the dentate gyrus (DG) and Cornu Ammonis 1(CA1) sub-regions of the hippocampus in mice brain (Fig.7.1, appendix). Studies herein also confirm that mice hippocampal neurons also express IL-1R1, IL-1RacP and MyD88 in culture (Chapter 5). This current study focuses on characterizing molecular mechanisms underlying IL-1β release and functions in moderation of E/I balance in hippocampus. It is therefore relevant to review endogenous expression of IL-1β ligand and its signaling components in CNS, particularly in hippocampus.

1.4 Neurobiology of Interleukin-1β

IL-1β is a well-characterized cytokine of the innate and adaptive immune system (Breder, Dinarello, and Saper 1988; Gibson, Rothwell, and Le Feuvre 2004; Allan, Tyrrell, and Rothwell 2005; Fogal, Hewett, and Hewett 2005) and is an important mediator in the communication between the peripheral immune and central nervous systems (CNS) (Hansen et al. 1998; Vitkovic et al. 2000;Samad et al. 2001; Patricia Parnet et al. 2002; Wolf et al. 2007; Liu et al. 2013). Within the CNS, while it contributes to the pathogenesis of various neuro-inflammatory and neurodegenerative maladies (Ho and Blum 1997; Allan, Tyrrell, and Rothwell 2005; Fogal, Hewett, and Hewett 2005; Simi et al. 2007; Shaftel, Griffin, and O'Banion 2008), it also modulates certain physiological functions (Fang, Wang, and Krueger 1998; Mason et al. 2001; Yirmiya, Winocur, and Goshen 2002; Avital et al. 2003; Goshen et al. 2007; de la Mano et al. 2007). Thus, IL-1β is a neuromodulator in both the normal and misfunctioning CNS.

1.4.1 IL-1β in neuroinflammation and neurodegeneration

IL-1β is a multifunctional proinflammatory cytokine (a protein released from/of immune cells that mediated immune functions). Initially described in the 1940s, as endogenous pyrogen, it is now associated primarily with innate immunity as a central mediator of endotoxic shock and in autoimmune diseases like Rheumatoid Arthritis and Multiple Sclerosis (Dinarello 2002; Daun and Fenton 2000). As an immune modulator, it is produced primarily in activated macrophages and lymphocytes of the immune system in response to infection, toxins and other inflammatory mediators [reviewed in (Mantovani et al. 2019)]. IL-1β has been a key player in different inflammatory and infectious conditions, including malignancies and transplantation, which are comprehensively reviewed by Dinarello et. al. (Dinarello 2002; Garlanda, Dinarello, and Mantovani 2013; Mantovani et al. 2019). IL-1β is a key pro-inflammatory player which has been

implicated by multiple researchers in several neuro-inflammatory and neurodegenerative conditions such as cerebral ischemia, traumatic brain injury, multiple sclerosis, excitotoxicity, epilepsy, Alzheimer's disease, Parkinson's Disease, etc. as reviewed in (Rothwell and Luheshi 2000; Gibson, Rothwell, and Le Feuvre 2004; Allan, Tyrrell, and Rothwell 2005; Simi et al. 2007; Fogal and Hewett 2008; Vezzani et al. 2011).

Inflammation in the central nervous system causes rapid induction in expression and release of several inflammatory mediators – cytokines, chemokines, prostaglandins, complement proteins, etc –leading to classic inflammatory response. In addition, increased permeability of the blood-brain barrier facilitates the invasion by peripheral immune cells (Hopkins and Rothwell 1995). Microglia are the primary source of inflammatory IL-1β in CNS (Perry and Teeling 2013; Liu and Quan 2018). Predominantly microglia and also astrocytes are the effector cells involved in acute and chronic neuroinflammation. Pathological concentrations of IL-1β activates the classical MAPKs/NF-κB signaling pathways in glial cells leading to cellular activation and production of various secondary mediators such as cytokines (IL-6, IL-8), adhesion molecules (I-CAM, V-CAM) and neuroprotective and neurotoxic factors (NGF, MMP- 9) (Simi et al. 2007). Simi et al. reviewed the cell-specific effects to IL-1β signaling in neuroinflammation and degenerative disorders. IL-1β promotes astrocyte proliferation and astrogliosis, upregulates adhesion molecules in endothelial cells to recruit leucocyte, promotes GABAergic inhibition and NMDAR phosphorylation in neurons. Therefore, IL-1β has different effector function in different cell types, inducing both toxic and protective functions, in response to neuroinflammation and degeneration (Simi et al. 2007).

IL-1β is connected to neuroinflammation by, i) level of IL-1β immediately rises following traumatic insult in the brain causing neuronal damage (Eriksson et al. 1999), ii) exogenous IL-1β

(cerebral injection) exacerbates ischemia, excitotoxicity and traumatic injury and iii) blocking IL-1β functions reduces neuronal injury (Eriksson et al. 2000), as summarized by Rothwell and Luheshi (Rothwell and Luheshi 2000). In the CNS, IL-1β often is associated with a bystander effect where immune responses lead to exacerbated neuronal tissue damage.

IL-1β in seizure and epilepsy: IL-1β is implicated in several acute seizure studies and studies involving the process of epileptogenesis (a progression by which brain achieves lowered seizure threshold and becomes susceptible to spontaneous seizure activity) utilizing different models of seizure and epilepsy.

IL-1β showed a rapid elevation in IL-1β mRNA in different parts of the brain following kainate (kainic acid/kainate- a potent neuroexcitatory amino acid agonist which acts by activating kainate receptors for glutamate) and PTZ induced model of the acute seizure (Minami et al. 1990). Kainic acid-induced model of seizure in several studies has shown induction of IL-1β mRNA (Yabuuchi , Minami, Katsumata 1993; Eriksson et al. 1999; 2000; Vezzani et al. 1999) along with increase in IL-1ra mRNA and protein (Eriksson et al. 1999; 2000). A different model of seizure, pilocarpine (a cholinergic agonist which activates muscarinic cholinergic receptors) induced *status epilepticus* also elevated IL-1β mRNA and protein (Voutsinos-Porche et al. 2004; Marcon et al. 2009) and IL-1R1 protein (Ravizza and Vezzani 2006a) in the brain. Status epilepticus induced by electrical stimulation in the hippocampus also increased expression of IL-1β mRNA and protein and its receptor (Ravizza and Vezzani 2006b).

Systemic IL-1β injection significantly increased PTZ induced seizure susceptibility (Miller and Turner 1990). Treatment with intrahippocampal IL-1β injection 10 min before kainate injection increased the duration of seizure, without affecting seizure latency or events (Vezzani et al. 1999). Here, exogenous IL-1β contributed to the maintenance of seizures in the hippocampus.

Exogenously administered IL-1ra via intra cerebrovascular injection significantly lowered electrically-induced seizure activity (De Simoni et al. 2000). On other hand, mice treated with IL-1ra before bicuculline treatment decreased seizure severity. Similarly, mice with overexpression of IL-1ra had less severe bicuculline-induced seizures (Vezzani et al. 2000). Caspase-1 activates IL-1β via proteolytic cleavage, therefore, blocking caspase-1 function would be presumed to reduce generation of the mature form of ligand, implicating IL-1β inactivity. Systemic administration of Caspase-1 inhibitor abrogated seizure activity in pertussis vaccine-induced seizure (Donnelly et al. 2001). Two caspase-1 inhibitors, Pralnacasan and VX-765, significantly increased seizure latency and decreased the number and duration of seizures when induced by intra-hippocampal kainate application (Ravizza et al. 2006; Maroso et al. 2011). Moreover, Caspase-1 null mice had increased seizure latency and less severe seizures compared to wild-type mice.

Our previous results demonstrated that the incidence of PTZ-induced convulsions and KA-induced *status epilepticus* was increased in mice in which IL-1β signaling was disrupted genetically (Claycomb, Hewett, and Hewett 2012), indicating role of endogenous IL-1β in the maintenance of seizure threshold.

IL-1β is also implicated in epileptogenesis or progression towards altered seizure threshold in the brain. IL-1β and IL-1RI mRNA rapidly increases in the cortex, amygdala, and hippocampus in rats with rapid electrical kindling of the amygdala (Plata-salaman et al. 2000). However, IL-1β transcript returned towards baseline after 3 weeks. IL-1β may be associated with kindling accusation but not maintenance. On the other hand, audiogenic kindling did not elevate IL-1β mRNA (Da Silva, Pereira, and Elisabetsky 1998).

Rats treated with IL-1β (i.c.v.) required more electrical stimulations of the amygdala to become kindled. Higher dose of IL-1β significantly decreased the severity and duration of kindled seizures in amygdala-kindled rats. IL-1β treatment retarded kindling development in a dose-dependent manner (Sayyah et al. 2005). Inhibition of IL-1β production via caspase-1 inhibitor prevented the rapid kindling of the hippocampus (Ravizza 2008). Continuous intra-cerebrovascular injection of IL-1β transiently delays the process of kindling compared to saline-treated rats whereas LPS treatment accelerated the process of PTZ-induced kindling of seizures (Kołosowska et al. 2014). Claycomb demonstrated no difference in PTZ-induced kindling in mice in IL-1R1 KO mice (Claycomb 2011).

While some evidence suggests that IL-1β possesses proconvulsant properties (Vezzani et al. 1999; 2000; Plata-Salamán et al. 2000; Heida, Moshé, and Pittman 2009; Maroso et al. 2011), other results are consistent with an anticonvulsive function of IL-1β (Miller and Turner 1990; Sayyah et al. 2005). This may be related, at least in part, to the model or approach used (Pinteaux, Trotter, and Simi 2009). However, the role of IL-1β in this brain disorder is complicated and depends on the disease model/paradigm utilized, microenvironment, and the cell type in question [reviewed in (Rijkers et al. 2009; Vezzani et al. 2013a; 2013b; Vezzani 2014)]. IL-1β is also implicated in febrile seizure (Dubé et al. 2005), however, it is not discussed in detail here.

Role of IL-1β in epilepsy remains paradoxical, however, it is important to recognize, firstly, role of IL-1β depends on the model of seizure or epileptogenesis, secondly, it is important to delineate role of endogenous v/s exogenous IL-1β, as the role of endogenous IL-1β in maintenance of the seizure threshold in normal brain (Claycomb, Hewett, and Hewett 2012), which when absent increased seizure severity.

1.4.2 IL-1β and its implication in CNS physiology

Interleukin-1β has physiological functions in CNS. The physiological role of IL-1β involves neurogenesis (Friedman 2005), the hypothalamus-pituitary axis of neuroendocrine functions (Watt and Hobbs 2000), fever response (Parnet et al. 2002; Nadjar et al. 2005), central stress axis (Goshen and Yirmiya 2009) and pain sensitization (Samad et al. 2001), sleep (Fang, Wang, and Krueger 1998), learning (Ross et al. 2003) and memory (Huang and Sheng 2010; Yirmiya and Goshen 2011).

These physiological functions of IL-1β in CNS are identified when its normal physiology is disrupted by disease or via application of exogenous IL-1β. For example, somnogenic function of IL-1β was identified with sickness behavior (Krueger 2008). IL-1β sits in between a complex balance of physiology v/s pathophysiology in CNS. The effector functions of physiological IL-1β depends on its concentration and the microenvironment within CNS. Increased IL-1β levels during neuroinflammation can affect these physiological functions. For example, long term potentiation was negatively impacted by inflammatory IL-1β levels (Vereker and Lynch 2000) and prolonged neuroinflammation in transgenic mice overexpressing IL-1β showed contextual memory impairment (Hein et al. 2012).

Some physiological functions of IL-1β that are associated with hippocampus are discussed here. Pertinent to this study, physiological functions of IL-1β associated with maintenance of E/I balance via synaptic regulation of neurotransmitters will be discussed.

Modulates neurogenesis: IL-1β is detected in the mammalian brain during the formation of the cortical plate (Dziegielewska et al., 2000). Its expression is also detected in late prenatal and early postnatal period. However, it is lower in adult CNS (Zunszain et al. 2012). During neurodevelopment, microglia-derived IL-1β acts as a mitogen for astroglia which in turn aids in

neurogenesis (Giulian et al. 1988). Adult neurogenesis in mice was affected in the DG of the hippocampus by IL-1β in IFN-γ induced clinical depression like behavior (Kaneko et al. 2006). Model of clinical depression mediated lowered neurogenesis was co-related with elevated IL-1β in the hippocampus via the kynurenine pathway (Zunszain et al. 2012). Mild stress reduced neurogenesis in mice which was absent in IL-1R1 KO mice (Goshen et al. 2008). Both acute and chronic exogenous IL-1β exposure reduced hippocampal neurogenesis (Goshen et al. 2008). On the other hand, IL-1ra overexpression lowered neurogenesis (Spulber 2008). IL-1β induces neurotrophic factors (Friedman 2005). Although exogenous acute IL-1β injection induced expression of neurotrophic factors and neuroprotection, sub-acute exposure to IL-1β caused opposite effect (Song, Zhang, and Dong 2013).

Sleep: Sleep is an important physiological function of the brain, that on the molecular level involves intricate neuronal functioning (Krueger et al. 2008). IL-1β increased non-rapid eye movement sleep (NREMS) whereas inhibition of IL-1β reduces spontaneous sleep (Krueger et al. 1998). Central administration of IL-1β increases NREMS and suppresses REMS (Opp, Obal, and Krueger 1991). In both cases, IL-1β levels were low and did not trigger fever response (Krueger et al. 1998; Opp, Obal, and Krueger 1991).

Mice lacking IL1R1 spend less time in NREMS under baseline conditions (Krueger et al. 1998; Fang, Wang, and Krueger 1998). IL1β is also known to induce fever response and thus implicated in fever associated sleep responses due to inflammatory challenges (Fang, Wang, and Krueger 1998; Taishi et al. 2012; Garlanda et al. 2013).

Synaptic plasticity: Synapses depending on the rates of their neuronal activity undergo molecular and sub-cellular changes (synaptic plasticity) to alter efficacy (synaptic strength). Long term potentiation is the biological process by which specific synaptic stimulation results in

long lasting increase in strength of synaptic transmission. Long term potentiation (LTP) which is considered to be the cellular equivalence of memory, is persistent augmentation of synaptic efficacy in CNS.

IL-1β mRNA level was elevated 1 hour after LTP in rat hippocampal slices and rat ipsilateral hippocampus with robust potentiation (Schneider et al. 1998). IL-1β mRNA level remained elevated up to 8 hours following induction of LTP (del Rey et al. 2013).

Exogenously applied IL-1β when applied 20 minutes before tetanic stimulation of rat mossy fiber path in hippocampal slices caused a reduction in LTP magnitude, but blocking IL-1β did not affect LTP (Katsuki et al. 1990). Similarly, IL-1β applied ten and sixty minutes before tetanus induced potentiation, attenuated the LTP in CA1 region of rat hippocampal slices (Bellinger, Madamba, and Siggins 1993). Recent study where 1ng/ml IL-1β was applied before thirty minutes of high-frequency stimulation in mouse brain slice showed impaired LTP in Schaffer collateral-CA1 synapses or the associational/commissural (A/C) fiber- CA3 synapses dependent on NMDA reception activation. However, NMDA independent pre-synaptic LTP was not impaired in mossy fiber-CA3 synapse showing the varied synapse-specific effect of IL-1β in the hippocampus of mice brain (Hoshino et al. 2017).

IL-1ra application 30 min after the induction of LTP in the DG of the hippocampus *in vitro* reduced synaptic activity back to baseline levels (O'Connor and Coogan 1999). In a separate study, when IL-1ra was applied 40 minutes before stimulation in a hippocampal slice, the initial increase in synaptic activity was not affected but was also not sustained and it subsided by thirty min (Ross et al. 2003).

LTP was not induced in Schaffer collateral *in vitro* or mossy fiber *in vivo* in IL-1R1 null mice (Avital et al. 2003). However, no impairment in LTP was observed in IL-1Ra or IL-1β KO mice (Ikegaya et al. 2003), posing the question of how IL-1β may signals in this model of LTP.

LTP in hippocampus can be affected by IL-1β by either of the following ways. Firstly, exogenous IL-1β inhibits LTP *in vitro* (Bellinger, Madamba, and Siggins 1993; Katsuki et al. 1990; Cunningham et al. 1996) or *in vivo* (Murray and Lynch 1998; Vereker and Lynch 2000). Secondly, effect of IL-1β is dose dependent (Schneider et al. 1998; Loscher, Mills, and Lynch 2003; Goshen et al. 2007; Spulber et al. 2009). Elevated concentrations of IL-1β inhibited induction of LTP (Katsuki et al. 1990; Bellinger, Madamba, and Siggins 1993; Lynch 2014) particularly in the hippocampus, including CA1 (Bellinger, Madamba, and Siggins 1993; Ross et al. 2003), CA3 (Katsuki et al. 1990), and dentate gyrus (Connor and Coogan 1999; Lynch 2014) and on the other hand, blocking endogenous IL-1β signaling with IL-1ra treatment attenuated maintenance of LTP (Ross et al. 2003). This indicated physiological IL-1β is necessary for induction and maintenance of LTP.

Learning and Memory: Hippocampus is the part of the brain associated with learning and a certain form of memory formation. As mentioned earlier, physiological IL-1β functions are highly prevalent in the hippocampus. Evidence indicates both beneficial and detrimental effects of IL-1β based on context and particular model of study. Administration of IL-1β (i.c.v) impaired hippocampal-dependent spatial memory (tested through performance in Morris water maze) (Yirmiya, Winocur, and Goshen 2002) and contextual fear conditioning in mice (Pugh et al. 2001) and rats (Hein et al. 2007). However, it had no impact on hippocampal independent process. IL-1Ra transiently reduced the underlying neuroplasticity changes. Inhibiting IL-1β signaling via IL-1ra administration or by IL-1R1 deletion also impaired hippocampal based

spatial memory and diminished fear conditioning memory in mice (Yirmiya 2002; Avital et al. 2003).

Stress or inflammation also affects learning and memory via IL-1β signaling. Bacterial lipopolysaccharide (LPS) injection impaired hippocampus-dependent contextual fear conditioning, memory in the passive avoidance paradigm, and deteriorates spatial learning in rats, and these effects were reversed by IL-1ra administration (Pugh et al. 1998). IL-1β mediated memory impairment is studied in different inflammatory conditions such as traumatic brain injury (Clausen et al. 2011).IL-1β causes cognitive decline with age as implicated in studies with patients with Alzheimer's disease (Lynch 1998). IL-1β level was elevated in older mice. It was implicated in cognitive decline (Gomes et al. 2013; Simen et al. 2011). However, these effects of IL-1β are affected by certain factors such as age (Bilbo et al. 2008; Takemiya et al. 2017), doses (Brennan, Beck, and Servatius 2003; Goshen et al. 2007) kind of memory studies and memory types [short term v/s long term (Spulber et al. 2009; Hein et al. 2012)], etc.

In summary, high doses of exogenous treatment of IL-1β or its elevation due to inflammation in the brain impairs hippocampus-dependent memory functions and on other hand inhibiting the signaling also causes similar impairment, indicating a physiological role of endogenous IL-1β in the maintenance of these memory functions.

Regulation of neurotransmission: IL-1β modulates both GABAergic and glutamatergic functions in CNS (Miller and Fahey 1994; Zhu et al. 2006).

Glutamate function: IL-1β facilitated the augmentation of neuronal calcium when induced by glutamate in hippocampal neurons (Wang et al. 1999). It increased the NMDA receptor-mediated current and the amplitude of the voltage-dependent Ca^{2+} current (Yang et al. 2005). Pretreatment of neurons with IL-1β enhanced NMDA induced rise in intracellular calcium and this effect was

abolished with IL-1ra treatment in cultured hippocampal neurons (Viviani et al. 2003). However, frequencies of spontaneous excitatory postsynaptic currents (sEPSC) and miniature excitatory postsynaptic currents (mEPSC) were decreased with the application of 10 or 100 ng/ml IL-1β, implying it acted presynaptically to decrease the frequency of neurotransmitter release. As the amplitude of mEPSC remained unchanged, it seemed unlikely that IL-1β acted on post-synaptic neurotransmitter receptor (Yang et al. 2005). The effect of IL-1β on the NMDA-induced outward currents in mechanically dissociated hippocampal neurons was studied using a perforated patch recording technique. Thirty (30)–hundred (100) ng/ml IL-1β inhibited the mean amplitude of the NMDA- induced outward currents. Hundred (100) ng/ml IL-1β also increased the mean ratio of the NMDA-induced inward current amplitudes significantly. This implied, IL-1β facilitated NMDA receptor mediated response (Zhang et al. 2008). IL-1β shares dynamic relationship with NMDA function. Treatment with NMDA (50μM for 10 minutes) on cultured hippocampal neurons, but not with IL-1β (0.05ng/ml for 30 minutes) led to a significant increase in the IL-1R1/GluN2B complex (GluN2B is a subunit of NMDAR) even though both treatments increased IL-1R1 in the synaptic sites (Gardoni et al. 2011).

GABAergic function: IL-1β application equivalent to pathophysiological conditions (1–10 ng/ml) decreased the peak magnitude of current elicited by 30μM GABA irreversibly, which was prevented by IL-1ra (Wang et al. 2000). IL-1β potentiated GABA mediated inward currents in chick cortical neurons and enhanced GABAergic signaling in slice preparation (Miller and Turner 1990). Alongside, IL-1β increased muscimol (GABA agonist) induced Cl^- uptake in synaptosome (Miller and Fahey, 1994). IL-1β enhances GABA-mediated inhibition within the hippocampus (Hellstrom et al. 2005).

Physiological role of IL-1β that encompasses hippocampal functions are discussed here. Beyond that, physiological role of IL-1β is also extended to fluid and hormone regulation in hypothalamus, effects on ion channel which in turn affects neuronal excitability (Schäfers and Sorkin 2008) and central axis of stress (Shintani et al. 1995; Goshen and Yirmiya 2009). Above mentioned studies identifying role of IL-1β in physiological function demonstrate a common theme, although physiological level of IL-1β is required for the specific function, excessive IL-1β impair the physiological function. Excessive IL-1β impaired synaptic plasticity and memory, affected synapse function which in turn affected E/I balance, although presence of IL-1β is required for maintenance of LTP, learning and memory and neurotransmitter functions. Physiological function of IL-1β in CNS therefore requires a fine balance (Pozzi et al. 2018).

1.5 IL-1β Synthesis, Processing, and Release

Transcription and translation: IL-1β gene has a TATA box within the promoter region alongside the cAMP-responsive element, an NF-kB binding site, AP-1 site, and Sp-1/PU binding site (Shirakawa et al. 1993). Transcriptional and translation regulation of IL-1β has been reviewed in (Fenton 1992; Allan, Tyrrell, and Rothwell 2005).

Processing and release: IL-1β processing and release is a multistep mechanism. It is produced as pro-peptide that must undergo proteolysis to form the mature peptide. There are several ways through which IL-1β can be processed within the cell and similarly can be released via different mechanisms. It's intracellular processing to its functional form requires help of a multimeric protein complex, inflammasome and proteolytic enzyme, Caspase-1, and activation of purinergic ATP receptor, P2X7R for its release outside the cell. Different cell types and stimuli can vary IL-1β processing time, subcellular localization and release mechanisms (Dinarello 2002; 2009).

Pro-IL-1β lacks a peptide leader sequence and therefore not processes via endoplasmic reticulum /Golgi pathway secreted outside the cell independent of the endoplasmic reticulum and Golgi apparatus (Rubartelli et al. 1990; Anelli and Sitia 2008). The primary source of IL-1β at sites of inflammation is the macrophage and processing and release mechanism of IL-1β in macrophage is extensively studied (Mankan et al. 2012; Ward et al. 2010; Pelegrin, Barroso-Gutierrez, and Surprenant 2008; Barbera-Cremades et al. 2012). In CNS, the IL-1β processing and release mechanism is studied in detail in microglia, which are the primary source of IL-1β in CNS during inflammation (Yao et al. 1992; Sanz and Virgilio 2000; Mingam et al. 2008; Bianco et al. 2005).

Firstly, ligand binding to a pattern recognition receptor (PRR) activates NF-κB to induce a 31 kD pro- IL-1β and NLRP, a key component of inflammasome, the multimeric protein complex required for Caspase-1 processing (Latz, Xiao, and Stutz 2013). Inflammasomes are large (~1μm) intracellular multimeric protein complexes that comprise a pattern recognition receptor (PRR), an adapter molecule and the enzyme caspase-1 amongst other proteins (Pétrilli, Papin, and Tschopp 2005). Secondly, assembly and activation of the inflammasome initiates a chain of events within the cell that catalyzes proteolytic cleavage of pro-IL-1β to 17kD mature peptide (Brough and Rothwell 2007). This step and subsequent release of IL-1β is triggered by P2X7R activation (Ferrari et al. 2006; Weber, Wasiliew, and Kracht 2010; Piccioli and Rubartelli 2013; Latz, Xiao, and Stutz 2013).

Mechanism of IL-1β release varies depending on cell types and conditions. Five different release mechanisms of IL-1β have been shown (1) exocytosis of IL-1β-containing secretory lysosomes where pro-IL-1β is cleaved by Caspase-1 in the lysosome, (2) release of IL-1β via shedding of plasma membrane microvesicles which is seen in microglia, (3) fusion of multivesicular bodies

with the plasma membrane and subsequent release of IL-1β-containing exosomes as seen in macrophage (Pelegrin, Barroso-Gutierrez, and Surprenant 2008) where inflammasome activation and caspase-1 are simultaneously required for IL-1β packaging, (4) export of IL-1β through the plasma membrane using specific membrane transporters under sustained NLRP3 activation, and (5) release of IL- 1β upon cell lysis, as reviewed in (Weber, Wasiliew, and Kracht 2010; Piccioli and Rubartelli 2013; Latz, Xiao, and Stutz 2013). The release of IL-1β from stimulated monocytes posits that extracellular ATP activates the purinergic P2X7 receptor, leading to processing and secretion of mature IL-1β (An and Wewers 2004). P2X7R is a member of the P2X family of ionotropic receptors of purinergic signaling. Upon engaging ligand, P2X7R permits the rapid efflux of K^+. This initiates activation of the inflammasome and three phospholipases, phosphatidylcholine-specific phospholipase C (PC-PLC), and calcium-independent (iPLA2) and calcium-dependent phospholipase A2 (cPLA2)., Calcium-independent phospholipase A2 is involved in processing whereas phosphatidylcholine-specific phospholipase C and calcium-dependent phospholipase A2 are required for secretion. The ATP-mediated K^+ efflux causes phosphatidylcholine-specific phospholipase C induction, which in turn allows the rise in intracellular free calcium concentration required for activation of phospholipase A2. This activation is ultimately responsible for IL- 1β secretion via any of the above-mentioned release pathways (Walev et al. 2000; Ferrari et al. 2006; Silverman et al. 2009) as seen in microglia (Sanz and Virgilio 2000) and macrophage (Pelegrin, Barroso-Gutierrez, and Surprenant 2008). Recently, it has been shown, Caspase-1 activation in macrophage in turn activates Gasdermin D (pore forming protein), which is involved in pyroptosis mediated IL- 1β secretion (Ramos-Junior and Morandini 2017; Monteleone et al. 2018). Non pyroptotic myeloid cells initially require Gasdermin D to be translocated to the plasma membrane for IL- 1β secretion initially, however,

later IL- 1β secretion is Gasdermin D independent. A very recent study have indicated role of Gasdermin in CNS, where its activation by Caspase-1 mediated enhanced cell permeability for IL-1β release from hippocampus in acute stress response (Frank et al. 2020).

1.5.1 Neurobiology of IL-1β release

Proteins required for inflammasome formation, Caspase-1 and P2X7 expression (Walsh, Muruve, and Power 2014; Mingam et al. 2008) are present in CNS particularly in microglia where it has been well studied. Proteins of the inflammasome (Savage et al. 2012; Hua et al. 2015; Wang et al. 2017), Caspase-1 (Denes, Lopez-Castejon, and Brough 2012) and P2X7R (Sperlágh and Illes 2014) have been implicated in both brain physiology and pathophysiology. The role of the inflammasome in CNS is reviewed in (Walsh, Muruve, and Power 2014). The neurobiology of P2X7R, which is required for activation of inflammasome of and IL-1β release, and therefore, pertinent to this thesis research, is discussed below.

1.5.2 P2X7R

P2X7R belongs to a large family of purinergic plasma membrane receptors (Burnstock 2008; Kumaria, Tolias, and Burnstock 2008; Tsuda, Tozaki-Saitoh, and Inoue 2012).P2X7R is ATP gated, nonselective cation channels, of ionotropic P2X receptors. The structural motif of P2X7R consists of two transmembrane domains (TM1, TM2), a short intracellular N- terminal domain, a large, glycosylated, cysteine-rich extracellular loop, and an intracellular C-terminal domain. Molecular physiology of P2X7R is reviewed in (Sperlágh and Illes 2014). P2X7R is expressed in a variety of different cell types including immune effector cells and cells of CNS. Apart from being a low-affinity receptor of ATP (which itself is a neurotransmitter in CNS apart from its normal role of being the source of energy), P2X7R also serves to release IL-1β from its cell (Kanellopoulos and Delarasse 2019; Miras-Portugal et al. 2017).

1.5.2.1 Neurobiology of P2X7R

P2X7R is a low-affinity ATP receptor associated with IL-1β release best studied in microglia and macrophages (Sanz and Virgilio 2000; Pelegrin, Barroso-Gutierrez, and Surprenant 2008). Endogenous P2X7R is expressed in various cell types of CNS including neurons, astrocytes and microglia (Sperlagh et al. 2006). Exact physiological expression profile of P2X7R in CNS particularly in neurons is ambiguous mainly due to issues with antibody specificity and presence of many alternative splice variant which produces different isoform of the receptor. These factors led to several issues of using commercially available antibodies to delineate the distribution of P2X7R expression due to pseudo-immunoreactivity in the P2X7R KO mice and several physiological stimuli causing changes to its expression (Sperlagh et al. 2006; Sperlágh and Illes 2014). GFP tagging downstream of P2X7R promoter clearly shows the expression of P2X7R expression in both neurons and microglia of the hippocampus, particularly in DG subregion (Engel et al. 2012). P2X7R modulates certain physiological function in CNS including synaptic functions (discussed further in 1.4.2.2), neurogenesis (Leeson et al. 2019), memory and learning (Labrousse et al. 2009; Domingos et al. 2018), and in the pathophysiology of neuroinflammation and neurodegeneration (Le Feuvre, Brough, and Rothwell 2002; Cotrina and Nedergaard 2009; Sperlágh and Illes 2014).

1.5.2.2 Relevance of P2X7R in E/I balance

The high expression levels of P2X7R observed in the hippocampus (Kamei et al. 2005; Engel et al. 2012; Sebastián-Serrano et al. 2016) occurs in excitatory nerve terminals and colocalized with VGLUT1 transporter of DG and CA3 region (Kamei et al. 2005). P2X7R is a low-affinity ATP receptor and has roles in neurotransmitter release and other synaptic function (Miras-Portugal et al. 2017). In particular, P2X7R activation regulates the release of both glutamate and GABA

(Papp, Vizi, and Sperlágh 2004; Kamei et al. 2005). Overexpression of the P2X7R in hippocampal brain slices decreases both sodium current amplitude and intrinsic neuronal excitability, whereas P2X7R inhibition has the opposite effect (del Puerto et al. 2015) implying P2X7R may modulate homeostatic E/I balance in the brain. P2X7R KO mice show impaired IL-1β expression in the hippocampus of mice brain, where the mice demonstrated impaired hippocampal based spatial memory (Labrousse et al. 2009).

P2X7R has been demonstrated to have paradoxical role in seizure and epilepsy (Engel et al. 2012). In patients with temporal lobe epilepsy, P2X7R antagonist, JNJ-47965567 suppressed epileptic seizures and reduced gliosis (Jimenez-Pacheco et al. 2016). P2X7R levels were elevated in intra-amygdala induced kainic acid injection, and seizure severity and hippocampal neuronal damage were reduced by pharmacological antagonism of P2X7R (Mesuret et al. 2014; Engel et al. 2012). Alongside, P2X7R antagonism (using pharmacological inhibitors - Brilliant Blue G, AFC-5128, JNJ-47965567 and Tanshinone IIA sulfonate) in rats showed that the mean seizure stage was lowered in PTZ induced kindling model than saline-treated rats. They also required more PTZ injection compared to saline-treated rats to reach their first convulsive seizure (Fischer et al. 2016). RNAi mediated P2X7R silencing in pilocarpine treatment reversed the increased edema in the hilus, dentate gyrus, CA1, and CA3 of hippocampus, reduced mortality rate following status epilepticus, increased the time to onset of a spontaneous seizure and reduced the number of seizures compared to only pilocarpine treated rats (Amorim et al. 2017). Inhibiting P2X7R activation had neuroprotective effects on the kainic acid mediated intra-amygdalar focal onset status epilepticus of seizure and epilepsy (Henshall and Engel 2015).

However, on the contrary, in rats, P2X7R antagonism showed no significant difference in seizure severity with PTZ induced acute seizure or differed in seizure threshold in maximal electroshock

seizure threshold test (Fischer et al. 2016). Alternatively, P2X7R KO mice showed elevated seizure susceptibility in pilocarpine-induced seizures (Kim and Kang 2011).

The evidence for its role in seizure and epilepsy is paradoxical.

1.6 COX-2 as a possible candidate of IL-1β signaling in the maintenance of neuronal excitation

IL-1β modulates its physiological and inflammatory functions m via several downstream mechanisms of which some of the important downstream mechanisms are induction of specific gene expression and synthesis of cyclooxygenase2 (COX-2), inducible nitric oxide synthase etc. causing PGE_2 production, platelet activation, and nitric oxide production, causing pain, vasodilation, hypotension and fever response (Dinarello 2002; 2009; 2018). In the following section, relationship between IL-1β and COX-2 is reviewed.

Several inflammatory functions of IL-1β are mediated via a potent lipid mediator called prostaglandin (PG). PG are derived from arachidonic acid. The first committed step in their synthesis is catalyzed by heme-containing bis-oxygenases, Cyclooxygenase (COX), officially known as prostaglandin-endoperoxide synthase (PTGS). It controls a large spectrum of functions, ranging from physiological activities, such as blood flow and gastric acid secretion to pathophysiological functions as the primary mediators of innate immunity working as one of the body's first lines of defense against infection and allergy (Ricciotti and FitzGerald 2011). Cyclooxygenases (COXs) are two isoforms, COX-1 and COX-2. COX-2 is one of the key candidates in CNS with a multitude of functionalities involving neuroinflammation and neurodegeneration including involvement in acute seizure and epilepsy (Hewett, Bell, and Hewett 2006). COX-2 has been seen to work downstream of IL-1β signaling in systemic inflammation including functions in CNS (Dinarello 2009; 2018). This section will describe the

role of COX-2 in CNS in relevance to my dissertation study, discuss link between COX-2 and IL-1β signaling and regulation of prostaglandin production via COX-2 downstream of IL-1β signaling which may affect the neuronal E/I balance.

1.6.1 Cyclooxygenase-2 (COX-2)

COX enzymes catalyze cellular reactions by which Arachidonic acid plus two molecules of O_2 are converted to ProstaglandinG_2 (PGG_2) and a peroxidase reaction via which PGG_2 is reduced to ProstaglandinH_2 (PGH_2).

Cyclooxygenase-1 (COX-1) is constitutively expressed throughout the body, whereas constitutive COX-2 expression is limited to certain tissues. Constitutive expression of COX-2 in CNS is found in certain subpopulations of glutamatergic neurons, but it is not constitutively detected in glial population (Hewett, Bell, and Hewett 2006). For this study, the primary focus will be on expression and functionalities of COX-2 in CNS particularly associated with neuronal hyperexcitation.

1.6.1.1 COX-2 expression in CNS

Basal expression of COX-2 mRNA and protein are observed in number of brain region, with higher expression levels in hippocampal and cortical neurons (Yamagata et al. 1993). Constitutive COX-2 expression in rodent brain was characterized by Breder et al (Breder, Dewitt, and Kraig 1995). It is primarily expressed by pyramidal neurons of the hippocampus, cortical layers 2/3, lateral amygdala, certain hypothalamic nuclei and in spinal dorsal and ventral horns and in the dorsal root ganglia, where it has been described to be present in the postsynaptic dendritic spine (Kaufmann et al. 1996; Chen, Magee, and Bazan 2002). Constitutive neuronal COX-2 expression is directly correlated with synaptic activity, thus suppressing glutaminergic

activity reduces COX-2 expression (Yamagata et al. 1993; Stark and Bazan 2011; Hewett et al. 2016).

Prostaglandins, lipid mediators produced from AA in COX dependent manner are expressed in different cell types of CNS, neurons (Taylor et al. 2008), astrocytes (Holgado et al. 2000; Takemiya et al. 2006), microglia (Pinteaux et al. 2002) including endothelial cells of the brain (Parfenova et al. 2002).

1.6.1.2 Role of COX-2 in CNS

COX-2 in CNS physiology has been implicated in the regulation of neuronal excitability, synaptic plasticity, pain sensitization and learning and memory. On the other hand, it plays a complex role in neuroinflammatory and neurodegenerative diseases including cerebral ischemia, multiple sclerosis, Parkinson's Disease, Alzheimer's disease and encephalopathies. This has been extensively reviewed by others (Hewett, Bell, and Hewett 2006; Yang and Chen 2008; Rojas et al. 2014). In the following section, I reviewed its role in acute seizure to better understand how IL-1β–COX-2 link may mediate E/I balance.

1.6.1.3 Role of COX-2 in acute seizure

Seizure activity increases COX-2 mRNA and protein in CNS (Okada et al. 2001; Takemiya et al. 2006; Tu and Bazan 2003; Voutsinos-Porche et al. 2004; Claycomb, Hewett, and Hewett 2011; Gong and Hewett 2018). Selective inhibition of COX-2 in seizure and epilepsy have yielded varied results, in some instances attenuating (Toscano et al. 2008) and in some other studies enhancing seizure response (Tu and Bazan 2003; Dhir and Kulkarni 2006; Dhir, Naidu, and Kulkarni 2006; Akula, Dhir, and Kulkarni 2008). Different inhibitors of COX-2 show different seizure responses in PTZ induced acute seizure. Rofecoxib (selective COX-2 inhibitor) in diet did not affect PTZ induced acute seizure but p.o rofecoxib treatment increased seizure severity in

mice (Claycomb, Hewett, and Hewett 2011; 2012). Study done in rats with blood brain barrier permeable COX-2 inhibitors, nimesulide (relatively COX-2 specific), celecoxib (selective COX-2 inhibitor) or etoricoxib (selective COX-2 inhibitor) treatment given 1 hour prior to PTZ injection showed only nimesulide attenuated PTZ induced seizure (Temp et al. 2017), showing the varied effect of COX-2 inhibitors on acute PTZ- induced seizures are influenced by timing and method of administration of drug.

Seizures are shown to induce prostaglandins within minutes in a COX-2 dependent manner (Zatz and Roth 1975; Förstermann et al. 1982; Akarsu, Mamuk, and Comert 1998; Kim et al. 2008; Yoshikawa et al. 2006) Many of these prostaglandins (PGE_2, PGD2, PGF2) are shown to have anticonvulsive properties (Förstermann et al. 1982; Akarsu, Mamuk, and Comert 1998; Kim et al. 2008). On the other hand, non-neuronal cells also produce late-onset prostaglandin in kainic acid-induced seizure model which when inhibited, blocked hippocampal neuron loss (Takemiya et al. 2006). Anti-PGE_2 antibodies attenuated PTZ-induced seizures in rats (Oliveira et al. 2008) indicating the pro-convulsive effects of prostaglandins.

Previous results from my lab indicate that inhibition of COX-2 markedly increased susceptibility of mice to seizure induction, suggesting that it may also contribute to the maintenance of the innate seizure threshold (Claycomb, Hewett, and Hewett 2012) and COX-2 overexpression increased seizure threshold (Gong and Hewett 2018). Role of COX-2 in seizure and epilepsy has is paradoxical.

1.6.2 IL-1β as an upstream modulator of COX-2 In CNS

Early indication of IL-1β working upstream of COX-2 came from a study done in rats showing that injection with recombinant IL-1β intra-peritoneally induced COX-2 mRNA in brain vasculature (Cao et al. 1996). Subsequent studies showed that exogenous application of IL-1β

increased COX-2 mRNA level and protein in primary hippocampal neuronal culture (Serou, Decoster, and Bazan 1999), dorsal root ganglion cells (Ohnishi et al. 2019a), neuroblastoma cells (Fiebich et al. 2000; Moolwaney and Igwe 2005), mice cortex (Moore, Olschowka, and O'Banion 2004) and in blood vasculature of mice hypothalamus and cortex (Dunn et al. 2006). IL-1β acting upstream of COX-2 has been shown to affect neuronal activity in cultured neurons and slice preparation. For example, exogenous IL-1β stimulated the release of substance P from cultured dorsal root ganglion (DRG) cells in a COX-2 dependent manner (Inoue et al. 1999). Additionally, depolarization of hypothalamic parvocellular and magnocellular neurons and hyperpolarization of GABAergic interneurons by exogenous IL-1β was blocked by selective COX-2 inhibition, indicating a role of COX-2 in IL-1β dependent changes in neuronal membrane potential (Ferri and Ferguson 2005). The effect of IL-1β on mEPSC amplitude was blocked by selective COX-2 inhibition (Sang et al. 2005).

IL-1β stimulation caused induction of COX-2 and subsequent PGE2 synthesis in murine astrocytes via Protein Kinase C and p38 and Extracellular signal-related Kinase (ERK1/2) of MAPK pathway activation (Molina-Holgado et al. 2000). IL-1β-mediated increase in slow-wave sleep (SWS) (hypothalamus driven sleep cycles) was blocked by the selective COX-2 inhibitors, piroxicam, and NS-398 (Terao et al. 1998). IL-1β-dependent induction of COX-2 contributes to hyperalgesia. In spinal cord neurons, central hyperalgesia due to peripheral inflammation was mediated by COX-2-dependent increase in PGE_2 which was blocked by intrathecal administration of IL-1ra (Samad et al. 2001). Alongside, the anti-convulsive effects of IL-1β in fully kindled rats were attenuated by the selective COX-2 inhibitor, piroxicam (Sayyah et al. 2005). Exogenous IL-1β induces COX-2 mRNA and PGE2 synthesis in cultured trigeminal ganglia neurons and glia caused neuronal sensitization to pain (Neeb et al. 2011).

Studies done till now and discussed here demonstrates role of exogenous application of IL-1β in inducing COX-2 expression and function. As present study focuses on endogenous neuromodulatory role of IL-1β, it is relevant to understand the difference between exogenous IL-1β - COX-2 link v/s physiological role of IL-1β and any possible link to endogenous COX-2 in CNS.

Rofecoxib, a selective inhibitor of COX-2, markedly enhanced the incidence of PTZ-induced acute convulsive seizures, suggesting that its activity contributes to the maintenance of the innate convulsive seizure threshold. Interestingly, rofecoxib did not enhance the susceptibility of IL-1R1 KO mice to the pro-convulsive action of PTZ, raising the possibility that the two pathways may be linked (Claycomb, Hewett, and Hewett 2012). No other studies are available to demonstrate possible link between endogenous IL-1β and COX-2. It will be investigated in this dissertation work.

1.7 Hippocampus – the region of interest

The hippocampal formation is a neuroanatomical structure of the limbic system of the brain which consists of intricate connections between sensory and motor cortices. It is primarily connected to the entorhinal cortex and is divided into the Dentate Gyrus(DG), the hippocampus proper (subdivided in Cornu Ammonis (CA) 1, 2, and 3), and the Subicular complex (subiculum, presubiculum, and parasubiculum). The hippocampus consists of unique and highly complex projection patterns from one sub-region to another (Witter 1993). Classically it is described as the unidirectional tri-synaptic excitatory neuronal circuit in the hippocampus. The primary input to the hippocampus initiates from the entorhinal cortex, primarily to the granule cells of DG and also to the pyramidal neurons of CA3 and CA1 via the Perforant Pathway. The granule cells project to the CA3 subregion via the Mossy Fibers. The pyramidal neurons of CA3 projects via

Association fibers to the contralateral CA3 and projects to the CA1 subfield via the Schaffer collaterals (Witter 1993; Strange et al. 2014). The neurons in subicular formation are the target for the pyramidal neurons of the CA1 subfield. The primary output from subiculum send projection back to layers 5 and 6 of the entorhinal cortex. Studies have shown complex and extensive networks of longitudinal and commissural projections are present in the hippocampus (Witter 1993).

In addition to its role in spatial processing and episodic memory it also contributes to emotions, motivation and in adult neurogenesis (Siebzehnrubl and Blumcke 2008; Knierim 2015). Hippocampus is highly affected in neuroinflammatory conditions like ischemia and temporal lobe epilepsy, neurocognitive deficit such as anterograde amnesia and vulnerable to neuropsychiatric diseases such as depression and schizophrenia (Strange et al. 2014; Knierim 2015). For studies herein, hippocampus was selected as region of interest as it is the possible site for studying neuromodulatory role of IL-1β in maintenance of seizure threshold. It also has relevance in context of maintenance of seizure threshold.

1.7.1 Hippocampus – Its importance in the context of seizure threshold

This section will elaborate the importance of hippocampus in context of physiological function - maintenance of seizure threshold and its relevance with seizure and epilepsy.

The hippocampus plays an important role in the process of seizure genesis and epileptogenesis as reviewed by (Sloviter 1994; Sutula et al. 1998; Ang, Carlson, and Coulter 2006; Fujita et al. 2014; Sloviter 2005). The DG, in particular, is an important control point for much of the information entering the hippocampus from the entorhinal cortex and it is thought to be involved in the reduction in seizure threshold in the epileptic brain (Gloveli, Schmitz, and Heinemann 1998; Sutula and Dudek 2007; Houser et al. 2012). Positive fMRI-BOLD signal analysis showed

that the DG was activated immediate prior to seizure onset (Brevard et al. 2006). Alongside, extreme susceptibility of the hippocampus to PTZ induced seizure is shown with an early immediate increase in c-Fos level and immediate increase in other neuropeptides (Yount, Ponsalle, and White 1994).

1.7.2 Hippocampus - The possible site of endogenous IL-1β signaling and function in murine brain

IL-1β is involved in several physiological activities or nervous system, particularly in regions of hypothalamus (fluid and hormone regulation, sickness behavior), hippocampus (long term potentiation, learning, and memory) and in spinal cord (pain sensitization and analgesia) (Summy-Long et al. 2006; 2008; Watt and Hobbs 2000; Schneider et al. 1998; Goshen et al. 2007; Wolf et al. 2007; Goshen and Yirmiya 2009; Lynch 2014).

IL-1β ligand and its receptor is constitutively expressed in hippocampus (Lechan et al. 1990; Kaneko et al. 2006; Viviani et al. 2014; French et al. 1999). P2X7R, the purinergic ATP receptor, implicated in IL-1β release is expressed in several cell types of brain and spinal cord (Sperlagh et al. 2006) and found to be involved in the physiological release of neurotransmitters in the hippocampus (Kamei et al. 2005). COX-2, identified as a possible downstream candidate for IL-1β in the maintenance of neuronal excitation, is constitutively expressed in CNS by CA3 neurons of the hippocampus (Yamagata et al. 1993) and induced in DG neurons via PTZ induced convulsive seizure (Claycomb et al. 2011).

Together, these studies support the importance of understanding the molecular mechanisms underlying the neuromodulatory roles of IL-1β in the hippocampus.

1.8 Specific aims

Studies in this dissertation work are focused on the investigation of the molecular mechanism of physiological IL-1β release in neurons and how it may contribute to the maintenance of the excitatory/inhibitory balance in hippocampal neurons. The following specific aims are hypothesized based on a previous study from our research group which showed neuromodulation by IL-1β signaling in the maintenance of seizure threshold (Claycomb, Hewett, and Hewett 2012). The goal of **Aim 1** is to confirm and extend previous studies implicating the role of endogenous IL-1β signaling in acute seizures (Chapter 2). The goal of **Aim 2** is to investigate the molecular mechanism governing physiological IL-1β release in the hippocampus and its role in the regulation of neuronal hyperexcitation. To investigate parallel aspects of this key question, this aim is divided further into *in vivo* and *in vitro* studies (Chapter 3). The goal of **Aim 3** is to investigate the possibility of effect of excitatory neuronal activity on IL-1β expression and signaling and subsequently analysis the possible link between neuromodulatory IL-1β signaling and downstream cyclooxygenase-2 (COX-2) expression and/or activity in the neurons of the hippocampus. This aim is also studied both *in vivo* and *in vitro* (Chapter 4). As primary hippocampal neurons were utilized for *in vitro* studies as mentioned in Chapter 3 and 4, details of the culturing procedure were documented in Chapter 5.

1.8.1 Specific Aim 1: *To demonstrate the reproducibility of the effect of IL-1RI gene deletion on the acute PTZ seizure response (chapter 2).*

For studies in Aim 1, the PTZ acute seizure model is used to corroborate the results demonstrating an increased seizure severity and incidence of convulsions in mice lacking IL-1 signaling capability. The hypothesis is that IL-1β contributes to the maintenance of seizure threshold and thus, more severe seizures will be elicited in mice lacking IL-1 signaling receptor. The study will also assess the difference in latency to PTZ induced convulsion and mortality associated with convulsions in between genotypes.

1.8.2 Specific Aim 2: *To investigate the molecular mechanism governing IL-1β expression and release in neurons of the hippocampus (chapter 3).*

Canonical processing and release of IL-1β protein is known to be ATP-dependent via P2X7 receptor (P2X7R) activation. Although studied in other cell systems, the mechanism of IL-1β release in neurons is yet to be understood. To assess this in mouse hippocampus, whether IL-1β release occurs through a similar mechanism, the brain permeable P2X7R antagonist, JNJ-47965567 will be given to CD-1 mice to determine if neuronal IL-1β release is blocked through immunohistochemical analysis. Secondly, using *in vitro* system (cultured hippocampal neurons), P2X7R dependent IL-1β release in the hippocampal neurons and its effect on excitatory neuronal activity will be studied using P2X7R antagonism. This system will also be exploited to investigate the subcellular location of IL-1β protein in the hippocampal neurons. Finally, seizure threshold will also be compared between mice dosed with the antagonist and the vehicle. Thus, these studies will allow us to determine i) whether P2X7R activation is required for constitutive IL-1β release, ii) to determine which neuronal populations may be responsible for

IL-1β production in hippocampus and iii) Role of P2X7R possibly via blocking of IL-1β release in modulation of the excitatory-inhibitory (E/I) balance both *in vivo* and *in vitro.*

1.8.3 Specific Aim 3: *To investigate the role of excitatory neuronal activity on IL-1β expression/signaling function in neurons of the hippocampus: possible link between IL-1β signaling and Cyclooxygenase-2 (COX-2) expression (Chapter 4).*

Specific Aim 3.1: To investigate if intensive neuronal excitation stimulates/alters IL-1β expression/signaling components in the mice hippocampus/hippocampal neurons.

The possibility of IL-1β release regulating changes in neuronal activity will be investigated in the prior chapters. This raises the possibility that excitatory neuronal activity may act as an endogenous stimulus for IL-1β mRNA and protein induction. To assess this *in vivo*, changes in IL-1β mRNA and protein, its signaling components in the hippocampus will be determined in a time-course study following acute convulsive seizures in male CD-1 mice. The activity-dependent changes in IL-1β protein expression will also be assessed in cultured hippocampal neurons.

Specific Aim 3.2: To investigate the possible link between IL-1β signaling and cyclooxygenase-2 (COX-2) expression and/or activity in neurons of the hippocampus.

Secondly, to understand if constitutive IL-1β signaling regulates COX-2 expression and/or activity in the hippocampal formation, constitutive/induced COX-2 expression and/or activity will be studied in mice genetically lacking the receptor, IL-1R1. It is hypothesized, if IL-1β signaling is involved in regulating neuronal COX-2 expression and/or activity in the hippocampus, COX-2 expression and/or activity will be attenuated or ablated in the hippocampal formation of mice lacking functional IL-1 signaling. Similarly, link between physiological IL-1β and COX-2 will be assessed *in vitro* using IL-1R1 antagonism in hippocampal neurons.

1.8.4 *Culturing murine primary hippocampal neurons (Chapter 5).*

A comparable system is required for the *in vitro* studies in chapter 3 and 4. Murine primary hippocampal neurons are cultured and maintained as a system to assess the molecular mechanisms associated with physiological IL-1β release and its neuromodulatory functions. As this system will be introduced in our laboratory system, detailed characterization will be done to understand and obtain comprehensive knowledge of this specific culture system and of the IL-1β ligands and it's signaling components *in vitro* to ensure success of the above-mentioned experimentation.

1.9 Significance

Epilepsy is characterized by recurrent seizures that result from abnormal synchronous firing of specific population(s) of neurons in brain (McNamara 1999; Hitiris 1996). Epilepsy is one of the most important brain diseases that occurs due to imbalance in homeostatic E/I balance. It is often associated with cognitive and learning deficits, psychosocial problems, and sudden unexplained death (Aarts, Binnie and Smit 1984; Hermann et al. 2001; Beghi et al. 2006; Surges et al. 2009). Although many current antiepileptic drugs affect excitatory or inhibitory neurotransmission directly, their use can be limited by adverse side effects. Moreover, epilepsy in a large number of patients is refractory to antiepileptic drug (Shorvon 1996; Löscher et al. 2009) making epilepsy difficult to treat and manage (Loscher et al. 2006; 2011). Therefore, researchers are in a constant look-out for new therapeutic targets. Along with several neurotransmitters in the brain which are affected by seizure and epilepsy, several neuromodulators have been identified which plays a role in the physiological regulation of these neurotransmitters and thus, indirectly in maintenance of physiological E/I balance. Therefore, these neuromodulators can also be critical therapeutic targets and should be well researched for their physiological and pathophysiological functions.

IL-1β signaling pathway has been widely studied and well-characterized primarily in neurodegenerative and neuroinflammatory diseases. Alongside, studies have also indicated several physiological roles of IL-1β in the CNS that are independent of its role as an immune modulator, including endogenous neuromodulatory role in the maintenance of E/I balance. Previous studies from our lab showed IL-1β regulates the innate seizure threshold, which arguably is a reflection of the homeostatic balance between excitation and inhibition (E/I) in the brain. However, much is unknown about its physiological cellular source(s), release mechanism and the functional significance of this release in maintenance of E/I balance. My dissertation studies will focus on these aspects. A better understanding of this endogenous neuromodulatory IL-1β signaling pathway in the brain that maintains the innate seizure threshold (physiological excitatory/inhibitory balance) may facilitate the development of novel therapies that may alter cellular pathways associated with seizures and epilepsy.

1.10 Details of common and experiment-based reagents required for the study.

Name of reagent (common)	Company	Catalog no.	Storage	Initial Conc.	Final conc.
Ultrapure water	Invitrogen	10977-015	Room temperature		
Ethanol	Pharmaco-AAPER	111000200	Room temperature		
Methanol	Avator Performance	3004-19	Room temperature		
Sodium azide (NaN3) 0.5% sol.	Aqua Solutions	8564	4°C	0.5 %	0.02%
PBS (*in vitro* use)	Invitrogen	21-040-CV	Room temperature		
Recombinant murine IL-1β	Peprotech Inc.	211-11B	4°C		

Name of reagent	Company	Catalog no.	Storage
Anesthesia			
Isoflurane	Henry-Schein Inc.	NDC11695-6776-2	Room temperature
Genotyping			
5X Green GoTaq Flexi Buffer (PCR reaction mix)	ProMega Corporations	M7122	-20°C
Deoxyribo-nucleotide triphosphate (dNTP)	ProMega Corporations	U1330	-20°C
DMSO	Fisher Chemicals	D128-500	Room temperature
$MgCL_2$	ProMega Corporations	A351H	-20°C
GoTaq polymerase	ProMega Corporations	M300D	-20°C
Agarose	Invitrogen (LifeTech)	16500-500	Room temperature
Ethidium Bromide	Omnipure(Calbiochem)	4410	Room temperature
RNA isolation and cDNA synthesis			
TriZol reagent	LifeTech (Ambion, RNA)	15596018	Room temperature
Isopropanol	Fisher Chemicals	BP2618-500	Room temperature
Chloroform	Fisher Chemicals	BP1145-1	Room temperature
1x M-MLV reaction buffer	ProMega Corporations	M1701	-20°C
oligo (dT) primers	ProMega Corporations	C110A	-20°C
dNTPs	ProMega Corporations	U1330	-20°C
Reverse transcriptase M-MLV	ProMega Corporations	M1701	-20°C
qPCR			
TaqMan™ Universal PCR Master Mix	Thermo Fisher	4304437	-20°C

Name of reagent	Company	Catalog no.	Storage	Concentration
Immunofluorescence				
Sucrose	EMD	SX1075	Room temperature	
Paraformaldehyde	EMD	19208	4°C	
Acetone	Fisher Chemicals	A18-1	Room temperature	
OCT	TissueTek	4583	Room temperature	
glass slides	FisherBrand Superfrost Plus	12-550-15		
Triton X	BioRad	161-0407	Room temperature	
Normal Goat serum	JAX Immuno Research	005-000-121	-20°C	60mg/ml
Normal Donkey serum	JAX immuno Research	017-000-121	-20°C	60mg/ml
Bovine serum albumin	JAX Immuno Research	001-000-161	4°C	
DAPI	Life technology	D-3571	-20°C	
Mounting media (Citifluor mountant solution)	EMS	AF1	Room temperature	
Coverslip	Corning	2955-245	Room temperature	
Protein estimation				
1X PBS	Gibco	21-040-CV	Room temperature	
BCA protein estimation kit	ThermoFisher	23225	Room temperature	
Western Blot				
Complete Protease Inhibitor	Roche	11836153001	4°C (aliquoted in -20°C)	
IodoA	Acros	12227-1000	4°C (aliquoted in -20°C)	
Mini Protean TGX Gel(4-15% gradient gel)	BioRad	456-1084	4°C	
All blue precision marker	BioRad	161-0373	-20°C	
Pink Blue precision marker	BioRad	161-0374	-20°C	
Running buffer(10X Tris/Glycine/SDS buffer)	BioRad	1610772	Room temperature	10X
Transfer buffer(10X Tris/Glycine buffer)	BioRad	1610771	Room temperature	10X
Nitrocellulose membrane	BioRad	162-0112	Room temperature	

LiCor Odyssey Blocking buffer(TBS)	LiCor	927-50000	4°C	
Tween 20	BioRad	170-6531	Room temperature	
Sodium dodecylsulphate	BioRad	161-0302	Room temperature	
ELISA				
Quantikine IL-1β ELISA kit	R & D systems	MLB00C	4°C	
Cell lysis buffer 2	R &D systems	895347	4°C	
Indomethacin	Sigma	18280	Room temperature	
PGE_2 ELISA kit	Cayman Chemicals	500141	-20°C	
EDTA(0.5M pH 8)	Invitrogen	AM9260G	Room temperature	
KH2PO4	EMD	PX1565-1	Room temperature	

Name of reagent	Company	Catalog no.	Storage
Pentylenetetrazol	Sigma	P6500	-20°C
JNJ-47965567 (*in vivo* use)	Tocris Biosciences	5299	4°C (dissolved in 30% SBE)
JNJ-47965567 (*in vitro* use)	Tocris Biosciences	5299	-20°C (stock solution)
Sulfobutylether-β-cyclodextrin	Medchem	HY-17031	Room temperature
Bicuculline methobromide	Enzo Life Sciences	BMLE A109-0050	-20°C (stock solution)
APV	Tocris Biosciences	106	-20°C (stock solution)
Y-VAD-CHO	Cayman chemicals	10014	-20°C (stock solution
NMDA	Sigma Aldrich	M3262	4°C (stock solution)
MK-801	Research Biochemicals	M107	-20°C (stock solution)

Table 1.2: List of reagents required for the dissertation research (with specifics).

Primary Antibody (IHC)	Host	Companies	Catalog no.	RRID no.	Initial Conc.	Dilution factor
IL-1β (H-153)	Rabbit	Santa-Cruz Biotechnology	sc-7884	AB_2124476	200 µg/ml	1:100
IL-1R1 (JAMA147)	Hamster	BioRad Laboratories	MCA2352GA	AB_2125035	1 mg/ml	1:100
P2X7R (Hano43)	Rat	BioRad Laboratories	MCA4713GA	AB_10547604	1 mg/ml	1:100
NeuN	Mouse	MilliporeSigma	MAB377	AB_2298772	1 mg/mL	1:100
P2Y12	Rabbit	Alomone Labs	APR012	AB_2040074	0.8 mg/ml.	1:200
COX-2	Rabbit	Cayman Chemicals	160106-1	AB_10077935	500 µg/ml	1:250
p-IRAK-1	Rabbit	Thermo-Fisher	PA5-38284	AB_2816657	1mg/ml	1:250

Primary Antibody (ICC)	Host	Companies	Catalog no.	RRID no.	Initial Conc.	Dilution factor
IL-1β (H-153)	Rabbit	Santa Cruz Biotechnology	sc-7884	AB_2124476	200 µg/ml	1:100
IL-1R1	Hamster	BioRad laboratories	MAB7711	AB_2125035	1 mg/ml	1:250
P2X7R	Rat	BioRad laboratories	MCA4713GA	AB_10547604	1 mg/ml	1:250
NeuN	Mouse	Millipore	MAB377	AB_2298772	1 mg/mL	1:100
MAP-2	Mouse	Thermo-Fisher Scientific	M1406	AB_477171	not provided	1:1000
Synaptophysin	Rabbit	AbCam	ab14692	AB_301417	0.65 mg/ml	1:500
Synaptophysin (D-4)	Mouse	Santa Cruz Biotechnology	sc-17750	AB_628311	200 µg/ml	1:500
PSD-95	Mouse	Thermo-Fisher Scientific	MA1-046	AB_2092361	1mg/ml	1:500
PSD-95	Goat	AbCam	ab12093	AB_298846	0.7mg/ml	1:500
IL-1RacP	Rabbit	Santa Cruz Biotechnology	sc-99031	AB_2125293	200 µg/ml	1:200
MyD88 (F-19)	Goat	Santa Cruz Biotechnology	sc-8197	AB_2146726	200 µg/ml	1:200
GABAaα/1	Rabbit	AbCam	ab33299	AB_732498	0.5mg/ml	1:500
GFAP	Mouse	Genetex	GTX73615	AB_378828	Not provided	1:50
COX-2	Rabbit	Cayman Chemicals	160106	AB_10077935	500 µg/ml	1:250

Secondary Antibody (IHC)	Host	Companies	Catalog no.	RRID no.	Initial conc.	Dilution factor
Anti Rabbit Alexa Fluor 488	Donkey	Jackson ImmunoResearch Labs	711-545-152	AB_2313584	7.5µg/ml	1:500
Anti Hamster DyLight 405	Goat	BioRad Laboratories	STAR104D405GA	AB_10846820	1 mg/ml	1:500
Anti Rat Dylight 549	Goat	BioRad Laboratories	STAR104D649GA	AB_10845149	1 mg/ml	1:500
Anti Mouse Alexa Fluor 350	Goat	ThermoFisher Scientific	A21049	AB_2535717	2mg/ml	1:500
Anti Mouse Fluorescein (FITC)	Goat	Jackson ImmunoResearch Labs	115-095-146	AB_2338599	7.5µg/ml	1:500

Secondary Antibody (ICC)	Host	Companies	Catalog no.	RRID no.	Initial conc.	Dilution factor
Anti Rabbit Alexa Fluor 488	Donkey	Jackson ImmunoResearch Labs	711-545-152	AB_2313584	7.5µg/ml	1:500
Anti Mouse Alexa Fluor 594	Donkey	Jackson ImmunoResearch Labs	715-585-150	AB_2340854	7.5µg/ml	1:500
Anti Mouse Alexa Fluor 488	Donkey	Jackson ImmunoResearch Labs	715-545-150	AB_2340846	7.5µg/ml	1:500
Anti Goat Alexa Fluor 594	Donkey	Jackson ImmunoResearch Labs	705-585-147	AB_2340433	7.5µg/ml	1:500
Anti Rabbit Alexa Fluor 488	Goat	Jackson ImmunoResearch Labs	111-545-144	AB_2338052	7.5µg/ml	1:500
Anti Hamster DyLight 405	Goat	BioRad	STAR104D405GA	AB_10846820	1 mg/ml	1:500
Anti Rat Dylight 549	Goat	BioRad	STAR104D649GA	AB_10845149	1 mg/ml	1:500

Antibody (for WB)	Host	Company	Catalog no.	RRID no.	Initial conc.	Dilution factor
IL-1β	Anti-Rabbit	AbCam	ab9722	AB_308765	1mg/ml	1:500
β-actin	Anti-Mouse	Sigma-Aldrich	A5441	AB_47644	Not provided	1:4000
LiCor Odyssey Anti Mouse IR dye 800 CW(for WB)	Goat	LI-COR Biosciences	926-32210	AB_621842	1mg/ml	1:10000
LiCor Odyssey Anti Rabbit IR dye 680 LT (for WB)	Goat	LI-COR Biosciences	926-68021	AB_10706309	1mg/ml	1:20000

Table 1.3. List of antibodies utilized in the dissertation study (with specifics).

Reagents	Company	Catalog no.	Storage
Ara-C	Sigma	C-6645	4°C (aliquoted in -20°C)
B-27(50X) (with AO)	Gibco	17504-055	-20°C
Beta-mercaptoethanol	Gibco	21985-023	4°C
Boric acid	EMD	BX0865-1	Room temp.
Cell culture grade H2O	Corning	25-055-CV	Room temp.
Glutamax	Gibco	35050-06	room temp.
Hanks Balanced Salt Solution	Corning	21-021-CV	Room temp.
HEPES	Corning	25-060-CL	Room temp.
Horse serum	Gibco	26050-088	4°C (aliquoted in -20°C)
L-glutamine	Gibco	25030-081	-20°C
Neurobasal A media	Gibco	10888-022	4°C
Penicillin	Gibco	15140-122	-20°C
Poly-L-lysine	Sigma	P9155	Room temp. (aliquoted in -20°C)
Sodium tetraborate	Sigma-Aldrich	221732	Room temp.
Streptomycin	Gibco	15070-063	-20°C
Trypan Blue	Mediatech Cellgro	25-900-CL	Room temp.
Trypsin	Mediatech Cellgro	25-050-CL	-20°C

Table 1.4: List of reagents required for the culturing neurons (with specifics).

qPCR probes(FAM-MGB)	Catalog no.	Company	Storage
IL-1β	Mm00434228_m1	Thermo Fisher Applied Biosystems	-20°C
IL-1R1	Mm00434237_m1	Thermo Fisher Applied Biosystems	-20°C
IL-1RaP	Mm00492638_m1	Thermo Fisher Applied Biosystems	-20°C
COX-2	Mm00478374_m1	Thermo Fisher Applied Biosystems	-20°C
P2X7R	Mm00440581_m1	Thermo Fisher Applied Biosystems	-20°C
Caspase-1	Mm00438023_m1	Thermo Fisher Applied Biosystems	-20°C
c-Fos	Mm00487425_m1	Thermo Fisher Applied Biosystems	-20°C
β-actin	Mm01205647_m1	Thermo Fisher Applied Biosystems	-20°C

Table 1.5: Details of qPCR probes for mRNA studies (with specifics).

Specific Aim 1: ***To demonstrate the reproducibility of the effect of IL-1RI gene deletion on the acute seizure response.***

Synopsis

Previous results from this research laboratory demonstrated that inactivation of the genes for interleukin-1β (IL-1β) or its signaling receptor sensitized mice to convulsant properties of pentylenetetrazol (PTZ), suggesting that constitutive presence of this cytokine contributes to the maintenance of the innate seizure threshold in the normal brain (Claycomb, Hewett, and Hewett 2012). This provided compelling evidence to support the premise of this dissertation that IL-1β functions as an endogenous neuromodulator that modulates neuronal activity in the brain that is associated with convulsive seizures. As this research investigated physiological localization of IL-1β in the hippocampus of murine brain and to characterize the role of IL-1β release in the regulation of the excitation/inhibition balance, it was essential to test the reproducibility of the primary concept on which the central hypothesis of the specific aims (Aims 2 and 3) are based.

Chapter 2

Effect of IL-1RI gene deletion on PTZ induced acute seizure response: Neuromodulatory role of IL-1β in maintenance of E/I balance - revisited.

Results from this chapter are part of the submitted manuscript**: P2X7-dependent constitutive Interleukin-1β release from pyramidal neurons of the normal hippocampus: Evidence for a role in maintenance of brain homeostasis.** Spandita S. Dutta, Torsten Wöllert, Sandra J. Hewett, and James A. Hewett. Manuscript submitted.

2.1 Summary

Interleukin-1β (IL-1β) is well-characterized as a proinflammatory cytokine. Within the central nervous systems (CNS), however, it is known to modulate certain physiological functions. In this regard, function of physiological IL-1β signaling in acute seizure was re-examined using mice with genetic disruption of IL-1β signaling receptor,IL-1R1, and modeling PTZ induced acute seizure. In the PTZ induced acute seizure model, the severity and incidence of acute convulsive seizures was higher in mutant Il1r1 (KO) mice compared to their respective wildtype littermates. This result reconfirmed previous finding from this lab indicating IL-1β possesses anti-convulsant properties and implying role of endogenous IL-1β in maintenance of seizure threshold.

2.2 Introduction

The role of IL-1β as a neuromodulator in seizure and epilepsy remains controversial. For example, while some evidence suggests that IL-1β possesses pro-convulsant properties (A Vezzani et al. 1999; 2000; Plata-Salamán et al. 2000; Heida, Moshé, and Pittman 2009; Mattia Maroso et al. 2011), other studies are consistent with an anticonvulsive function of IL-1β (Miller and Turner 1990; Sayyah et al. 2005). This may be related, at least in part, to the model or approach used (Pinteaux, Trotter, and Simi 2009). Of particular relevance to this dissertation, our previous results demonstrated inactivation of the genes for interleukin-1β (IL-1β) or its signaling receptor, IL-1R1, sensitized mice to convulsant properties of pentylenetetrazol (PTZ), suggesting that constitutive release of this proinflammatory cytokine contributes to the maintenance of the excitatory-inhibitory (E/I) balance in the normal brain (Claycomb, Hewett, and Hewett 2012). Thus, *the goal here is to confirm and extend previous studies implicating endogenous IL-1β signaling in acute seizures.*

2.3 Materials and methods

2.3.1 Mice

2.3.1.1 Maintenance: Colonies of mutant IL-R1 mice in C57BL/6J background (Maliszewski et al. 1997) were established from breeders obtained from The Jackson Laboratory (Stock #003245). All mice were housed on a 12-hour light/dark schedule in the AAALAC-accredited laboratory animal care facility at Syracuse University. Cages contained Enrich-o'Cob bedding (The Andersons Lab Bedding Products, Andersons Inc.) and provided one-pass, low-velocity, total volume filtered air exchange under direct exhaust ventilation (OptiMICE®, Animal Care Systems, Inc.). The environment of the facility was controlled for temperature and humidity and standard mouse chow and water were provided *ad libitum*. Animal procedures were conducted in accordance with the National Institute of Health (NIH) guidelines for the use of experimental animals and were approved by the Syracuse University Institutional Animal Care and Use Committee (IACUC). All mice were handled for mock injections for 7 days prior to the performance of the actual experiment to habituate the mice to handling during injection mechanism.

2.3.1.2 Breeding: Male (10-15 weeks old) wildtype (WT or +/+) and mutant (KO or -/-) littermates for studies were derived from filial heterozygous (Het or +/−) breeding units that were obtained from parental crossing WT females with male KO mice. Heterozygotic and female offspring from these crossings were removed at weaning and male +/+ and -/- littermates were housed 3-4 per cage until they reached adulthood, to have a similar representation in each cage. The above breeding and caging strategy followed a previous study (Claycomb, Hewett, and Hewett 2012) to control for potential non-specific differences in environmental or genetic factors.

2.3.2 Genotyping

2.3.2.1 Tail biopsies and DNA extraction: Mice pups were weaned between 21-28 days of birth (Animal protocol submitted and approved by IACUC, Syracuse University, protocol no #14-013 and 17-010), their ear punched for identification and tail biopsied for genotyping. DNA from the tail biopsies were extracted using 180µl lysis buffer (25mM NaOH and 2mM EDTA) at 95°C for 30 min and neutralized with 180µl neutralization buffer (40mM Trizma base) and stored at 4°C for short term storage prior to use.

2.3.2.2 PCR: All genotyping was performed through PCR analysis of tail genomic DNA samples using allele-specific primers [(50µM stock), Integrated DNA technologies)]. PCR was run following **Protocol 27489** (The Jackson Laboratory) with mixing 5µl DNA and 20µl PCR master mix (Table 2.1). DNA sequence of the allele-specific primer were, common, 5'-CCG AAG AAG CTC ACG TTG TCA AG-3'(forward) and wildtype specific 5'-GAG TTA CCC GAG GTC CAG TGG-3' (backwards) and knock out (KO) or mutant specific 5'-GAA TGG GCT GAC CGC TTC CTC-3' (backwards).

WT reaction Master mix	Initial conc.	Amount in one reaction (ml)		KO reaction Master mix	Initial conc.	Amount in one reaction (ml)
Ultrapure H_2O		10		Ultrapure H_2O		9.75
PCR reaction mix	5X	5		PCR reaction mix	5X	5
Primers (WT+all)	5mM each	2		Primers (KO+all)	5mM each	2
Deoxyribo-nucleotide triphosphate	100mM	0.5		Deoxyribo-nucleotide triphosphate	100mM	0.5
DMSO		0.25		DMSO		0
$MgCL_2$	25mM	2		$MgCL_2$	25mM	2.5
Taq polymerase	50 units/ml	0.2		Taq polymerase	50 units/ml	0.2

Table 2.1: Details of components of PCR Master Mix

2.3.2.3 Agarose gel electrophoresis: The PCR product was then visualized using agarose gel electrophoresis. 2% agarose gel was made in 1X TAE buffer (50X TAE buffer stock was made with 242g tris base, 14.6 EDTA, 57.1ml glacial acetic acid, and volume made up to 1-liter ultrapure water) and 1µl ethidium bromide was added to the 50ml gel to detect DNA. 10µl of DNA was loaded in each lane and ran with DNA Kb ladder and pre-determined known samples of WT, KO, and het samples. Electrophoresis was conducted at 90V for 30 min and the gel is photographed with the Licor documentation system LiCor Odessey Fc (OFC-0116) at 600nm.

2.3.3 Pentylenetetrazol induced acute seizure paradigm

Pentylenetetrazol is a Gamma amino butyl acid (GABA) A receptor antagonist that triggers neuronal excitation indirectly through disinhibition. Pentylenetetrazol (PTZ) was aliquoted in dark tubes and kept in -20°C freezer in boxes with desiccant for long term storage. Before use, an aliquot of PTZ is brought to room temperature, weighed and dissolved in 0.9% saline, steriled through syringe filter and administered intraperitoneally (i.p.) in a volume of 0.3ml/0.003kg and seizure behavior scored using our 5 point severity scale as described previously: 0, normal behavior; 1, hypo-mobility; 2, myoclonus; 3 and 4, convulsion without and with loss of righting, respectively (Claycomb, Hewett, and Hewett 2011). Additionally, convulsion latency measured in seconds (time for onset of convulsive behavior), the incidence of convulsion (% of mice exhibiting convulsion, score ≥3), and mortality (% of mice exhibiting convulsion associated death) were also quantified for this study.

2.3.3.1 Dose-response study: A limited dose-ranging study was performed in C57BL/6J wild-type male mice to determine the dose of PTZ for studies with this strain. Male wildtype C57BL/6J mice were injected with a single dose of either 42, 43.5, or 45.5 mg/kg PTZ.

2.3.3.2 Acute seizure paradigm: Based on results from the dose-response analysis performed, acute seizure activity was induced by dose of 43.5 mg/kg PTZ (this dose yielded a spread of seizure response in WT mice) on C57BL/6J IL-1r1 WT and KO mice. Studies were performed without the genotype of the mice to ensure unbiased scoring (Fig.2.2).

2.3.4 Statistical analysis

All statistical analyses were performed using GraphPad Prism (ver. 8.0.2, GraphPad Software, Inc.). All tests are indicated in the result section and/or legends to the figure. Statistical significance was maintained at $p<0.05$. Behavioral data associated with scoring of seizure were reported as median seizure score and incidence of a convulsive seizure. Latency to convulsion after acute PTZ injection between genotypes was assessed using a two-tailed Mann–Whitney test. ROUT method of outlier test was used to detect outliers for latency to convulsion after acute PTZ injection between genotypes. Datasets representing proportions (incidence of convulsions) were analyzed using a two-tailed Fisher's exact test. Significance was set at $p< 0.05$. In figure legends, *p* values were reported as their exact values whenever available.

2.4 Results

2.4.1 Dose-response study

A dose-response study was performed to assess the acute PTZ-induced seizure response in C57BL/6 mice prior to proposed experiments. Cohorts of wild-type male C57BL/6 mice were treated with either of 3 different PTZ doses, 42, 43.5, or 45.5 mg/kg PTZ (Fig.2.1) and seizure behavior were scored using our 5-point severity scale as described previously. The dose that generated a spread of seizure response (23%) and the dose at which 100% mice had convulsive seizure were 43.5 and 45.5 mg/kg PTZ, respectively (Fig. 2.1). The dose of 43.5 mg/kg PTZ with a median score of 2 was selected for the study herein.

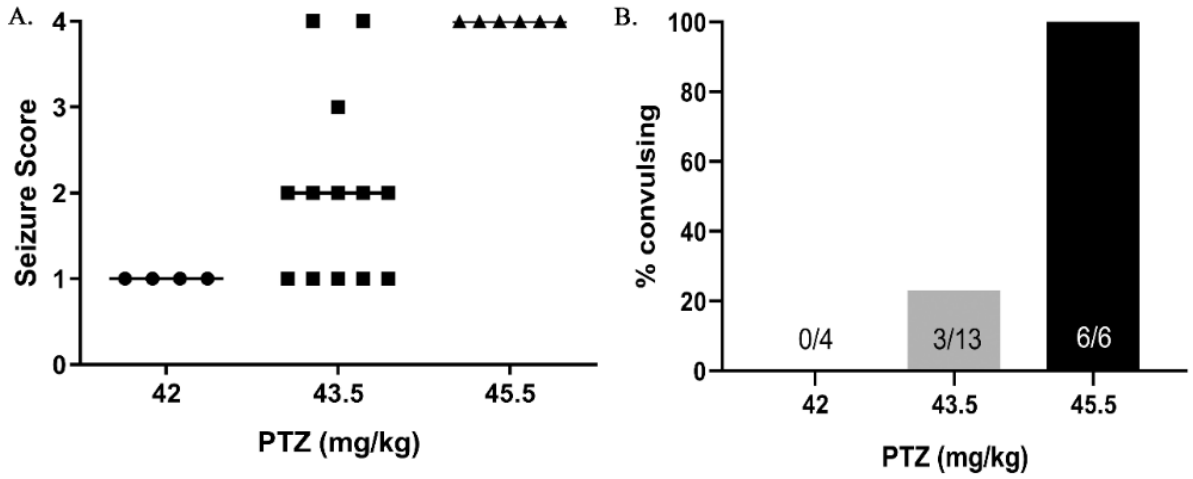


Fig 2.1. PTZ elicits seizure behavior in a dose-dependent manner.

Dose-response analysis of C57BL/6J WT male mice were conducted by treating mice with either 42mg/kg (N=4), 43.5mg/kg (N=13) or 45.5mg/kg (N=6) b.w.

A. Seizure severity. Each point on the graph is the seizure score for each mouse. The median seizure scores for 42mg/kg, 43.5mg/kg or 45.5mg/kg PTZ are represented by horizontal lines, respectively.

B. Incidence of convulsions. The number of mice exhibiting a convulsive seizure (PTZ seizure score≥3) in A expressed as a % of total mice injected with PTZ for each treatment group (ratio in the bar is the raw data).

2.4.2 Effect of IL1R1 gene deletion on the innate seizure threshold

A previous study reported that mice lacking IL-1 signaling exhibited enhanced sensitivity to pentylenetetrazol (PTZ) (Claycomb, Hewett, and Hewett 2012). To confirm this herein, wild type (+/+, WT) and Il1r1 mutant (-/-, KO) littermate mice were challenged with 43.5 mg/kg PTZ and seizure responses were assessed (Fig.2.2). This PTZ dose induced a median seizure severity score of 2 in wild type mice and 4 in I1r1 mutant littermates (**, p= 0.0035, Mann Whitney Test, two-tailed) (Fig.2.2A). KO mice showed corresponding higher incidence of convulsive seizure (67%) having convulsive seizures compared to WT littermate controls (38%) (*, p= 0.0026, 2x2, two-tailed Fisher's exact test) (Fig. 2.2C).

The mutant mice exhibited a shorter latency to convulsion (Fig. 2.2B). A significant difference was detected in mean latency to achieve convulsive seizures between the genotypes (*, p = 0.0094, Mann Whitney Test, two-tailed). Finally, while the number of mice that succumbed to convulsions was not significantly different between genotypes, it was nonetheless elevated in mutant mice (Fig. 2.2 D).

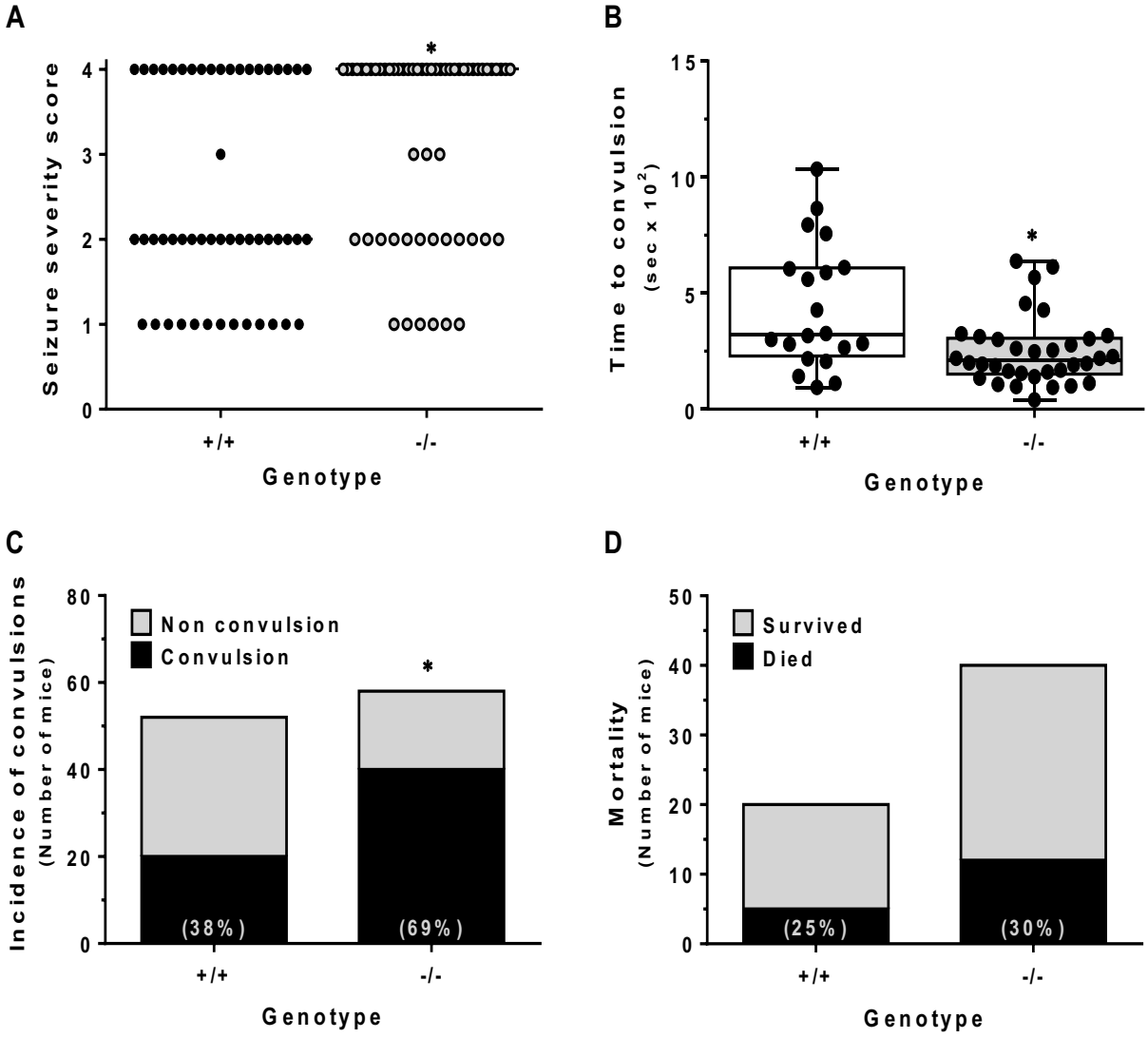


Fig.2.2. The innate seizure threshold is reduced in mice lacking IL-1 signaling.

Wildtype (WT) (+/+, N = 52) and Il1r1 mutant (KO) (-/-, N = 58) mice were treated with 43.5 mg/kg PTZ and seizure behavior was scored on a 5-point scale of increasing severity as described in methods (0 = normal behavior).

A. Seizure severity. Each symbol shows the maximum seizure score for individual mice (*, $p = 0.0035$, two tailed Mann Whitney Test). Horizontal line represents median seizure score.

B. Latency to convulsion. The ROUT method (Q = 1%) was performed to test for outliers and resulting data are expressed as individual latencies from mice with convulsive seizure in A, superimposed over a box and whisker plot. Analysis was performed on log transformed data (*, $p = 0.0041$, unpaired two tailed t-test).

(continued)

C. Incidence of convulsions. Data are expressed as the number of mice exhibiting convulsive seizure responses (score ≥3) and non-convulsive seizure responses (score ≤2) from A. The incidence of convulsions is expressed as % of the total population (*, $p = 0.002$, two tailed Fisher's exact test).

D. Mortality. Data are expressed as the number of mice that survived or died from convulsive seizures. The incidence of mortality is expressed as % of the total population ($p = 0.77$, two tailed Fisher's exact test).

2.5 Discussion

The neuromodulatory role of IL-1β signaling in the maintenance of seizure threshold in the PTZ model of acute seizure is confirmed. The previous study from our laboratory has demonstrated that the PTZ dosing that elicited median seizure score of 1 (floor effect) was 24mg/kg and median seizure score of 4 with PTZ causing a ceiling effect in seizure response was 43mg/kg (Claycomb, Hewett, and Hewett 2012), compared to the study herein, where similar seizure response was generated at 42 mg/kg and 45.5mg/kg, respectively. The median effective dose (intermediate population response) for the current study generated median score of 2 with a spread of seizure response is 43.5mg/kg which is higher than previously observed 36mg/kg PTZ. Several factors may contribute to the shift in the acute PTZ dose responses. Firstly, to factor differences in the physical environment, these mice were housed in a vivarium at Syracuse University as compared to University of Connecticut vivarium, for the previous study. Though mice are maintained on a similar light/dark schedule, specific conditions like caging, bedding etc. differed in these two vivariums. Secondly, several recent studies have shown that the sensitivity of mice to specific treatments varies between experimenters (Sorge et al. 2014; Georgiou et al. 2018). These factors may have contributed to this shift in the responsiveness of the mice towards higher concentrations of PTZ and a steeper dose-response curve compared to the previous researcher in the seizure response study of the mice that genetically lacked IL1r1. However, irrespective of dose used in either studies (36mg/kg v/s 43.5mg/kg), both studies show an identical phenotypic behavioral response, and it eliminates any question of lack of reciprocation of behavioral studies and strengthens this data which forms the basis of the overall hypothesis of this dissertation work.

Specific Aim 2: ***To investigate the molecular mechanism governing IL-1β expression and release in neurons of the hippocampus.***

Synopsis

While researchers have investigated about the cell types from which IL-1β is secreted and its molecular mechanism release of at sites of inflammation both in peripheral system and in central nervous system, nothing is known about its physiological release in the brain, specifically in the hippocampus, although there are clear indications of its distinct physiological function in CNS. The hypothesis of this aim is to investigate the molecular mechanism governing IL-1β release in neurons of the hippocampus. Specifically, its purpose is to determine if physiological IL-1β release may be dependent on the purinergic ATP receptor, P2X7R activation. It was reasoned that the brain-permeable P2X7 receptor antagonist, JNJ-47965567 (Bhattacharya et al. 2013) would result in IL-1β accumulation in the cells releasing it. If so, it would permit identification of the cellular source in the hippocampus under physiological conditions. This process will also be applied *in vitro*, to further examine the sub-cellular localization of IL-1β in hippocampal neurons. Finally, it was posited that the P2X7 receptor antagonist may alter excitatory neuronal activity *in vitro* and may produce a similar seizure phenotype in mice as seen in mice genetically lacking IL-1 signaling which exhibited enhanced sensitivity to convulsive stimuli (demonstrated in chapter 2) which will also be examined. Thus, studies from this section will assign an effort to provide insight into the molecular mechanism of physiological IL-1β release and further support for the notion that endogenous IL-1β contributes to the maintenance of the homeostatic excitatory/inhibitory balance.

Chapter 3

P2X7-dependent constitutive Interleukin-1β release from pyramidal neurons of the mouse hippocampus: Role in maintenance of E/I balance

This chapter is near duplicate of the submitted manuscript**: P2X7-dependent constitutive Interleukin-1β release from pyramidal neurons of the normal hippocampus: Evidence for a role in maintenance of brain homeostasis.** Spandita S. Dutta, Torsten Wöllert, Sandra J. Hewett, and James A. Hewett. Manuscript submitted.

3.1 Summary

Inactivation of the genes for interleukin-1β (IL-1β) or its signaling receptor, IL-1R1, sensitized mice to convulsant properties of pentylenetetrazol (PTZ), suggesting that constitutive release of this proinflammatory cytokine contributes to the maintenance of the excitatory-inhibitory (E/I) balance in the normal brain. However, the cellular and molecular mechanisms that control this homeostatic function remain to be clarified. This study focused the hippocampus because it is known to be an important site of constitutive IL-1β release and function. The purpose of this study was to determine whether the purinergic receptor, P2X7 is necessary for basal release of IL-1β in the normal hippocampus, and to test its role in maintenance of E/I balance. This was examined *in vivo* and in cell culture using JNJ-47965567 (JNJ), a highly selective antagonist of P2X7. Focus was IL-1β immunoreactivity was not detectible in the normal hippocampal formation under basal condition. However, an increase in immunoreactivity was observed in the pyramidal cells of the CA3 and to a lesser extent in the CA1 regions 60 minutes after treatment of mice with 30 mg/kg JNJ, suggesting that the antagonist blocked release IL-1β from these cells. No immunoreactivity was detected in granule cells of the dentate gyrus without or with JNJ treatment. In cultures of hippocampal neurons, IL-1β immunoreactivity was similarly increased by JNJ treatment and this was paralleled by a concomitant increase in the excitation-coupled genes, c-Fos and COX-2. Importantly, pretreatment with the P2X7 antagonist sensitized mice to the PTZ-induced convulsive seizure response in a manner that resembled genetic deletion of IL-1β signaling. Together, results from this report concludes that constitutive IL-1β release from hippocampal pyramidal neurons contributes to the homeostatic balance between excitation and inhibition in the normal brain. This function has implications for normal brain function and for epilepsy, a neurological disorder in which the seizure threshold is compromised.

3.2 Introduction

Interleukin-1β (IL-1β) is a well-characterized cytokine signaling molecule of the innate and adaptive immune systems (Dinarello 2009). It is expressed at high levels by infiltrating myeloid cells at sites of tissue damage or infection where it serves as an important mediator in the inflammatory response. Systemically, it is a mediator of the sickness behavioral response, thus functioning as a link between the peripheral immune and central nervous systems (CNS) (Hansen et al. 1998; Laye et al. 1995; Hosoi, Okuma, and Nomura 2000a; Wieczorek and Dunn 2006). Within the CNS, IL-1β has been implicated in the pathogenesis of various disease states (Rothwell and Luheshi 2000; Simi et al. 2007; Fogal and Hewett 2008), including cerebral ischemia, traumatic brain injury, epilepsy, Alzheimer's disease, and multiple sclerosis, although its function appears to be complex (Shaftel, Griffin, and O'Banion 2008; Pinteaux, Trotter, and Simi 2009; Hewett, Jackman, and Claycomb 2012). Remarkably, it is now clear that IL-1β modulates certain physiological functions in the CNS that are independent of its role as an immune modulator (Rothwell and Luheshi 2000; Hewett, Jackman, and Claycomb 2012). Among these are non-REM sleep (Krueger et al. 1998; Fang, Wang, and Krueger 1998; Opp, Obal, and Krueger 1991), body fluid regulation (Diana et al. 1999; Summy-Long et al. 2006; 2008) and long-term potentiation and memory (Schneider et al. 1998; Ross et al. 2003; Goshen et al. 2007). Pertinent to the current study, evidence suggests that constitutive IL-1 signaling contributes to regulation of the innate seizure threshold (Claycomb, Hewett, and Hewett 2012), a property of the normal brain that reflects the homeostatic control of neuronal excitation. Consistent with its modulatory role in the normal brain, constitutive expression of IL-1β and its signaling receptor, IL-1 receptor type 1 (IL-1R1), have been reported in the brain regions that subserve its physiological actions (Breder,

Dinarello, and Saper 1988; Lechan et al. 1990; Cunningham et al. 1992; Deyerle et al. 1992; Yabuuchi et al. 1994; Diana et al. 1999; Watt and Hobbs 2000; Friedman 2001; Liu et al. 2019). IL-1β is produced as an inactive 31kD protein (pro-IL-1β) that requires cleavage to form the active 17 kD peptide (Weber, Wasiliew, and Kracht 2010; Afonina et al. 2015). Since IL-1β lacks a leader sequence, its secretion occurs via a non-classical mechanism (Anelli and Sitia 2008; Piccioli and Rubartelli 2013). The canonical release mechanism occurring under inflammatory conditions requires two signals: an initial priming signal followed by a secondary activation and release signal. Several exogenous and endogenous factors can prime cells for IL-1β release, including bacterial products and pro-inflammatory cytokines, respectively. These molecules induce the expression of pro-IL-1β and NLRP3, a key component of the IL-1β processing inflammasome (Agostini et al. 2004; He, Hara, and Núñez 2016; Swanson, Deng, and Ting 2019). The secondary activation signal induces assembly and activation of the inflammasome leading to caspase-1-dependent (formerly Interleukin-1 Converting Enzyme) processing and subsequent release of the active IL-1β peptide (Swanson, Deng, and Ting 2019). Secondary signals are cellular stress factors that include extracellular ATP, an agonist of the purinergic ionotropic receptor, P2X7 (Dubyak 2012). While much remains unknown about the processing and release of IL-1β in the normal brain, some aspects may have similarities with its release at sites of inflammation. In this regard, administration of a potent ATP analog directly into the normal hippocampus induced the release of IL-1β, suggesting that its release in this region can occur by an ATP-P2X7-dependent mechanism in the absence of neuroinflammation (Bhattacharya et al. 2013).

P2X7 mRNA expression has been reported throughout the mouse brain in neurons as well as glia (Metzger et al. 2017). Expression was particularly prominent in the CA3 region of the

hippocampus, where it was localized to the excitatory pyramidal neurons. By comparison, expression was much lower or not detected in the other primary neuronal populations of the hippocampal formation, including the granule cells of the dentate gyrus. To confirm this expression profile, Metzger et al. inserted a human P2X7 cDNA transgene into the mouse allele, deleting all mouse transcript variants while retaining the innate transcriptional control mechanisms to drive expression of the transgene. Expression of the human P2X7 mRNA paralleled the innate mouse mRNA. This pattern of P2X7 expression in the hippocampus largely agrees with the in situ hybridization analysis provided by the Allen Brain Atlas [https://mouse.brain-map.org/experiment/show/81600560, Allen Mouse Brain Atlas, (Lein et al. 2007)]. P2X7R protein expression was studied for this report, which indicated its physiological expression in both neuronal and non-neuronal cells of hippocampus (detailed in Appendix section, Fig 7.2 & Fig. 7.3). Together with the evidence for P2X7-dependent release of IL-1β in the hippocampus (Bhattacharya et al. 2013), these results raise the possibility that CA3 neurons may be an important site of constitutive IL-1β release in the hippocampus. On the other hand, other evidence suggests that P2X7 protein is expressed on the presynaptic terminals of the mossy fiber synapses (Armstrong et al. 2002). This, together with the reported IL-1β immunoreactivity in mossy fibers (Lechan et al. 1990), argues that the granule cell population may be the cellular source of IL-1β in the hippocampus. Additional studies are necessary to distinguish between these two possibilities.

The highly selective, brain permeable P2X7 antagonist, JNJ-47965567 [JNJ, (Bhattacharya et al. 2013)], was used herein to examine further the cellular source of IL-1β in the normal hippocampus under basal conditions. It was reasoned that if IL-1β were constitutively released in the hippocampus via a P2X7-dependent mechanism, the antagonist would result in its

accumulation in the cells that release it. It was further posited that depletion of extracellular IL-1β by the P2X7 antagonist would lower the seizure threshold much like disruption of IL-1 signal transduction (Claycomb, Hewett, and Hewett 2012), thus further substantiating the role of basal IL-1β release in maintenance of the normal excitatory-inhibitory balance in the brain.

3.3 Methods

3.3.1 Mice

3.3.1.1 CD-1 mice: Male 6-7-week-old CD-1 mice for *in vivo* studies were obtained from Charles River Labs (Wilmington, MA) and housed three per cage for at least one week prior to use. All *in vivo* studies were performed using 8-12-week-old male mice, which were handled daily for seven days prior to treatments. For studies using hippocampal neuron cultures, pregnant female CD-1 mice were purchased from Charles River Labs and housed singly upon arrival at Syracuse University.

3.3.1.2 Animal housing: described in section 2.3.1.1 of chapter 2.

3.3.2 Mouse studies

3.3.2.1 JNJ-47965567 treatment: CD-1 mice were treated with the P2X7 receptor antagonist, JNJ-47965567 (JNJ), either alone or 60 minutes prior to induction of acute seizures. JNJ was dissolved in 30% Sulfobutylether-β-cyclodextrin (SBE) w/v water and injected subcutaneously at a dose of 30 mg/kg (Bhattacharya et al. 2013). Comparisons were made to control mice treated in parallel with the SBE-β-CD (SBE) vehicle solution.

3.3.2.2 Pentylenetetrazol induced acute seizure paradigm: Acute seizure activity was induced with pentylenetetrazol, a GABAA receptor antagonist used previously to assess alterations in the

innate seizure threshold in the brain of mice (Claycomb, Hewett, and Hewett 2011). PTZ injection solutions preparation, injection and scoring was done following methods explained section 2.3.3 of chapter 2. For studies in this chapter, 1 hour after s.c. injection of JNJ or its vehicle, PTZ is given intraperitoneally. Doses of PTZ are mentioned in the result section.

3.3.2.3 Anesthesia: For studies using mice, fully anesthetized mice [with 120 mg/kg ketamine, 20 mg/kg xylazine, intraperitoneally in saline (1X)] were exsanguinated and perfused transcardially with PBS. P0-P1 pups for hippocampal neuronal dissection were anesthetized using isoflurane (1 ml on a gauze pad in a closed container for approximately 8 pups).

3.3.3 Primary hippocampal neuron cultures

3.3.3.1 Preparation: Cultures were established using a protocol that was derived from previous reports (Ma et al. 2003; Uliasz et al. 2012; Beaudoin et al. 2012). The method of culturing hippocampal neurons has been broadly discussed in chapter 5 of this dissertation thesis.

Tissue culture plates or 6-well glass bottom culture plates for higher resolution microscopy were prepared by covering each well with 0.1M borate buffer (pH 8.5) containing 30µg/ml high molecular poly-*L*-lysine. After at least one hour at room temperature, the plates were washed thoroughly and dried under sterile conditions. Postnatal day 0-1 CD-1 mice were euthanized using isoflurane, then placed on ice and dissected under aseptic conditions. Hippocampi from 8-9 brains were pooled in sterile calcium and magnesium-free Hank's balanced salt solution containing 2.5g glucose, 3.5g sucrose, 2.4g HEPES (pH 7.4) and 0.25% trypsin (dissection medium, DM). After incubating briefly at 37^{o}C, the tissue was triturated gently and the dissociated cells pelleted and resuspended in Neurobasal A media containing 7% horse serum, 1% B27 supplement, 0.5mM L-glutamine, 1% of pen-strep and 5µM β-mercaptoethanol (2-ME). The cell suspension was added to a PLL-coated 24 well plate (0.4ml/well) and incubated at 37^{o}C

under a humidified atmosphere of 5% CO_2. The plating medium was replaced 12-16 hours later with medium consisting of Neurobasal A media containing 3% horse serum, 1% B27 supplement, 0.5mM of L-glutamine, 1% pen-strep, and 5 μM β-ME. Finally, this was replaced 6-8 hours later with serum-free growth medium (Neurobasal A media containing only 2% B27 supplement, 0.5mM glutamine, 1% pen-strep, and 5mM β-ME). To reduce contamination by glia and other non-neuronal cell types, the cells were treated with 6mM cytosine β-D-arabinofuranoside hydrochloride from 3-6 days in vitro (DIV). Cells were fed twice weekly by replacing 50% of the growth medium with fresh medium. This procedure yielded >90% enriched neuronal cell cultures.

3.3.3.2 Cell culture studies: All studies with primary hippocampal neuron cultures were performed at 14-15 days *in vitro* (DIV).

JNJ: For studies with JNJ, a stock solution of 2mM was prepared in DMSO and stored at -20°C. This solution was diluted in growth medium and administered to cells at final concentrations of 0.1 or 0.3μM.

Bicuculline: For studies with bicuculline methobromide, a 100mM stock solution was prepared in DMSO and frozen at -20°C. This was diluted in growth medium and administered to cells at a final concentration of 100μM.

APV: For studies with 5-amino-phosphovaleric acid (APV), a 1.2mM stock solution was prepared in water and frozen in aliquots at -20°C. This was diluted in growth medium on the day of use and administered to cells at a final concentration of 30μM.

3.3.4 Immunohistochemistry

3.3.4.1 Tissue collection, fixation and sample preparation: For studies using mice, fully anesthetized mice were exsanguinated and sequentially perfused transcardially with phosphate-buffered saline (PBS) and 4% phosphate-buffered paraformaldehyde (PFA). The fixed brains were excised from the cranium and post-fixed for several hours at 4°C in 4% PFA. The tissue was then saturated with 20% sucrose solution at 4°C, immersed in OCT reagent, snap-frozen on dry ice, and stored at -80°C until processing for IHC. Frozen brains were cut at -20°C on a cryostat (Thermo Fisher Scientific Microm HM550) to obtain 14 µm coronal sections, which were mounted on pre-coated slides and stored at -20°C.

3.3.4.2 Immunofluorescence: For staining, sections from treated mice were processed in parallel with their respective controls. Sections were bathed in PBS containing 0.25% Triton-X100 (PBST) and, depending on secondary antibody, incubated in 5% normal donkey serum solution containing 1% bovine serum albumin. Subsequently, sections were incubated overnight at 4°C with primary antibody in PBST containing 1% normal serum and 1% BSA, then washed thoroughly with PBS and incubated with species-specific secondary antibodies at room temperature in the dark. Sections were washed again with PBS, incubated for 5 min with PBS containing 1µg/mL DAPI, and mounted with coverslips using anti-fade mounting solution (Citifluor AF1). Images for each study were captured using an upright Zeiss Axio Imager A2 microscope fitted with an X-Cite 120Q fluorescence light source (Lumen Dynamics), AxioCam MRc digital camera, and ZEN 2 software (Version 2.0.0.0, Carl Zeiss Microscopy GmbH). For comparisons, images from each study were processed identically using Adobe Photoshop.

3.3.5 Immunocytochemistry

3.3.5.1 Cell fixation and sample preparation: For studies using hippocampal cultures, media was aspirated from culture wells and cells were incubated with 50/50 v/v methanol/acetone solution for 15 min and washed with PBS.

3.3.5.2 Immunofluorescence staining: The PBS is aspirated out of the well and incubated for 7 min with 0.25% Triton-X100 in PBS (PBST). Next, cells were incubated with 10% normal donkey in PBS for 1 hour at room temperature and subsequently incubated overnight at 4°C with primary antibody in PBS containing 2% normal serum in PBS. Wells were washed thoroughly with PBS and incubated with secondary antibodies for 30 min at room temperature in the dark. Images were captured using an inverted IX50 Olympus microscope equipped with an X-Cite 120Q fluorescence light source (Lumen Dynamics), Olympus D73 digital camera, and CellSens Standard software. For high resolution analyses, images were acquired with Zeiss Axio Observer Z1 inverted microscope equipped with 40X/1.30 oil Plan-Neofluar objective illumination using HAL100 12V 100W halogen lamp housed with microscope power supply (PhotoFluor LM-75, 89 North) and Hamamatsu CMOS ORCA-Flash4.0 LT CCD camera (C11440-42U30). For comparisons, images from individual studies were processed identically using Adobe Photoshop.

3.3.5.3 Antibodies for IHC and ICC.

Primary: A rabbit polyclonal anti-**IL-1β** IgG was used at 1:100 for staining brain sections and hippocampal neuron cultures (H-153, Santa Cruz Biotechnology, Cat #sc-7884, 200µg/mL, RRID:AB_2124476). The specificity of this antibody was validated using an IL-1β blocking peptide (Peprotech, Cat #211-11B) as described in Appendix, Fig.7.4 & 7.5 .

NeuN, MAP-2 (details are provided in table 1.3).

Secondary: Donkey anti-rabbit Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 594 (details are provided in table 1.3).

3.3.6 Quantification of IL-1β immunoreactivity.

3.3.6.1 For IHC studies: All brain section images of subregions of hippocampi were converted to 8-bit gray scale and fluorescent intensity was quantified using NIH FIJI (Image J2). The maximum and minimum thresholds were set to 255 and 31, respectively, using the default auto thresholding technique. Results from two images from each brain section were averaged and the mean fluorescent integrated density was calculated in the hippocampal CA3 and CA1 pyramidal cell layers using methods described previously (Gong and Hewett 2018).

3.3.6.2 For ICC studies: To quantify fluorescence intensity in cell bodies of hippocampal neurons in culture, z-stack images were summed through z-project and displayed as 2D maximum projection using ImageJ. For measuring fluorescence intensity of cell body, 6 steps were summed for maximum intensity (1 step size =100nm). Using FIJI, an outline was drawn around each cell (10 cells per field, 3 fields/well and 2replicates/treatment) and the area and mean fluorescence intensity was recorded. Adjacent background readings/fields were also taken. The corrected total cellular fluorescence (CTCF) = integrated density – (area of selected cell × mean fluorescence of background readings) was calculated and mean CTCF was compared between treatments using a protocol that was derived from previous report (McCloy et al. 2014). To measure fluorescent intensity in the neuronal processes, 8 steps were summed (MAP-2 co-stained) and displayed as 2D maximum projection using FIJI. Fluorescent intensity plot profile was calculated for 31 pixels (1 μm) along the processes approximately 0.5 μm away from cell body (10 neurites/microscopic field image, 5 field/well and 2 replicates/treatment), with point 0 being towards and 30 away from the cell body. Neurites arising from clearly identifiable neurons

were selected for the dataset. Mean pixel intensity profile was plotted and compared between treatments.

3.3.7 Quantitative PCR analysis

3.3.7.1 Sample collection: The growth medium was aspirated from 24 well culture plates and 0.250 mL of TriZol reagent was added to each well. Four wells were pooled per sample in centrifuge tubes and frozen at -80ºC until processing for quantitative PCR analysis.

3.3.7.2 RNA extraction: Centrifuge tubes containing samples in TriZol are thawed and 200ml of chloroform was added and mixed well. The tubes were then centrifuged at 10000*g for 15 min at 4°C to separate the aqueous layer. 500ml isopropanol was added to the separated aqueous layer and incubated for 10 min at room temperature. The mixture was then centrifuged at 10000* for 15 min at 4°C to pellet RNA. The RNA pellet was further washed in 75% ethanol to purify the RNA. After that, the pellet was resuspended in 20ml ultrapure water and heated at 70°C for 5 min and placed on ice. RNA obtained was then quantified using a NanoDrop UV-Vis Spectrophotometer (BioRad).

3.3.7.3 cDNA synthesis: cDNA synthesis was performed in a 21mL reaction mix containing 10ml 1mg/ml total RNA and 11ml master mix containing:

Reaction Master mix	**Amount in one reaction (µl)**
Ultrapure H_2O	2
1x M-MLV reaction buffer	4
oligo (dT) primers	2
dNTPs	1
Reverse transcriptase M-MLV	2

Table 3.1: Reagents in cDNA synthesis master mix

The reaction mixture was heated to 42°C in a heat block for 1 hour and then stored at -20°C for immediate use or -80°C for long term storage.

3.3.7.4 Quantitative PCR: Expression of c-Fos and COX-2 was quantified with a real-time PCR detection system (CFX Connect, BioRad Laboratories) using Applied Biosystems TaqMan Universal PCR Master Mix and FAM-MGB dye gene expression assay probes. Relative expression was quantified using the comparative cycle threshold method, ΔΔCt (Livak and Schmittgen 2001), where individual Ct values were normalized to the β-actin Ct value from the same sample then with a control sample Ct value (calibrator) to determine the relative fold changes in mRNA. The Applied Biosystems Gene expression assay probes are cFos, COX-2 and β-actin (details are provided in table 1.5).

3.3.8 IL-1β Immunoblot analysis

After aspirating growth medium from wells, cells were washed with PBS, harvested, then pooled (4 wells/sample in PBS) and pelleted at 2000g for 5 min at 4°C. Pellets were resuspended in RIPA buffer containing 50mM Tris, pH 7.5, 0.5% Na Deoxycholate, 0.1% SDS, 1%Nonidet P40, 150mM NaCl, 0.5M Iodoacetamide and 1X Complete protease inhibitor for 30 min on ice, after which lysates were cleared by centrifugation at 10,000*g for 20 min at 4°C. Total protein concentration was determined using a commercially-available kit then adjusted to 1μg/μL protein with 25% 4X SDS loading buffer and 30μg of lysate protein were separated on a 4-15% SDS-polyacrylamide gradient gel along with all Blue and Dual Color precision molecular size ladder. The separated proteins were transferred to nitrocellulose membranes, which were blocked for 1 hour in LiCor Odyssey blocking buffer in Tris buffer saline and incubated with rabbit anti-IL-1β polyclonal antibody, [Appendix, Fig.7.4 indicates this antibody detects both pro- and active IL-1β] and mouse monoclonal anti-β-actin antibodies overnight at 4°C. The immunoblots were washed with TBS containing 0.2% Tween20 and incubated with IRDye 680LT goat anti-rabbit IgG and IRDye 800CW goat anti-mouse IgG. After incubating for two hours at room

temperature on a rocker, the immunoblots were washed and imaged with a Licor documentation system (Odyssey Fc OFC-0116). Antibody details were provided in table 1.3.

3.3.9 Statistical analysis

Data were analyzed using GraphPad Prism, Version 8.4.1 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). Statistical tests are described in figure legends. Statistical significance was set to $p<0.05$.

Statistical tests are described in figure legends. Behavioral data associated with scoring of seizure are reported as median seizure score and incidence of a convulsive seizure. Latency to convulsion after acute PTZ injection between genotypes was assessed using a two-tailed Mann–Whitney test. Datasets representing proportions (incidence of convulsions) were analyzed using a two-tailed Fisher's exact test. For q-PCR, statistics were performed on the logarithmic transformation of $2^{-\Delta\Delta CT}$ values, and analyzed with Kruskal Wallis test followed by uncorrected Dunn's test for JNJ study or one-way ANOVA followed by uncorrected Fisher's LSD test for bicuculline and APV treatment study. One-way ANOVA was followed by uncorrected Fisher's LSD test and two-way ANOVA test was followed by either Tukey's multiple comparison or Fisher's uncorrected LSD test as deemed fit. For one-way ANOVA and two-way ANOVA test, in addition to *p* values, F values were determined for equal variance. (F (DFn, DFd) where DFn is the numerator of df (degree of freedom) and DFd is the denominator of df, in addition to *p* values).

3.4 Results

3.4.1 Effect of P2X7 antagonist on IL-1β release from mice hippocampus

Results in Chapter 2 imply that IL-1β is released constitutively in the normal brain to perform its neuromodulatory functions. To assess the cellular source of IL-1β in the hippocampus, CD-1 mice were treated with the blood brain barrier permeable P2X7 antagonist, JNJ-47965567 (JNJ), and results were compared to mice treated in parallel with its vehicle, SBE-β-CD (SBE). No immunoreactive IL-1β was detected in any region of the hippocampal formation of vehicle-treated mice (Fig. 3.1A-C). In contrast, it was detected in mice after exposure to the P2X7 antagonist for 60 minutes (Fig. 3.1 D-F). IL-1β immunoreactivity was strongest in pyramidal neurons of the CA3 region (Fig. 3.1 A/D), where it was approximately 5 to 10-fold higher than vehicle-treated controls (Fig. 3.2). Immunoreactivity was also detected in neurons of the CA1 region after antagonist treatment (Fig 3.1 B/E), albeit at much lower levels than the CA3 (Fig. 3.2). On the other hand, no immunoreactivity was detected in the granule neurons of the dentate gyrus (Fig. 3.1 C/F).

This study provided evidence of physiological build-up of IL-1β in CA3 and CA1 sub-region of hippocampus and also associating the requirement of P2X7R in its physiological release.

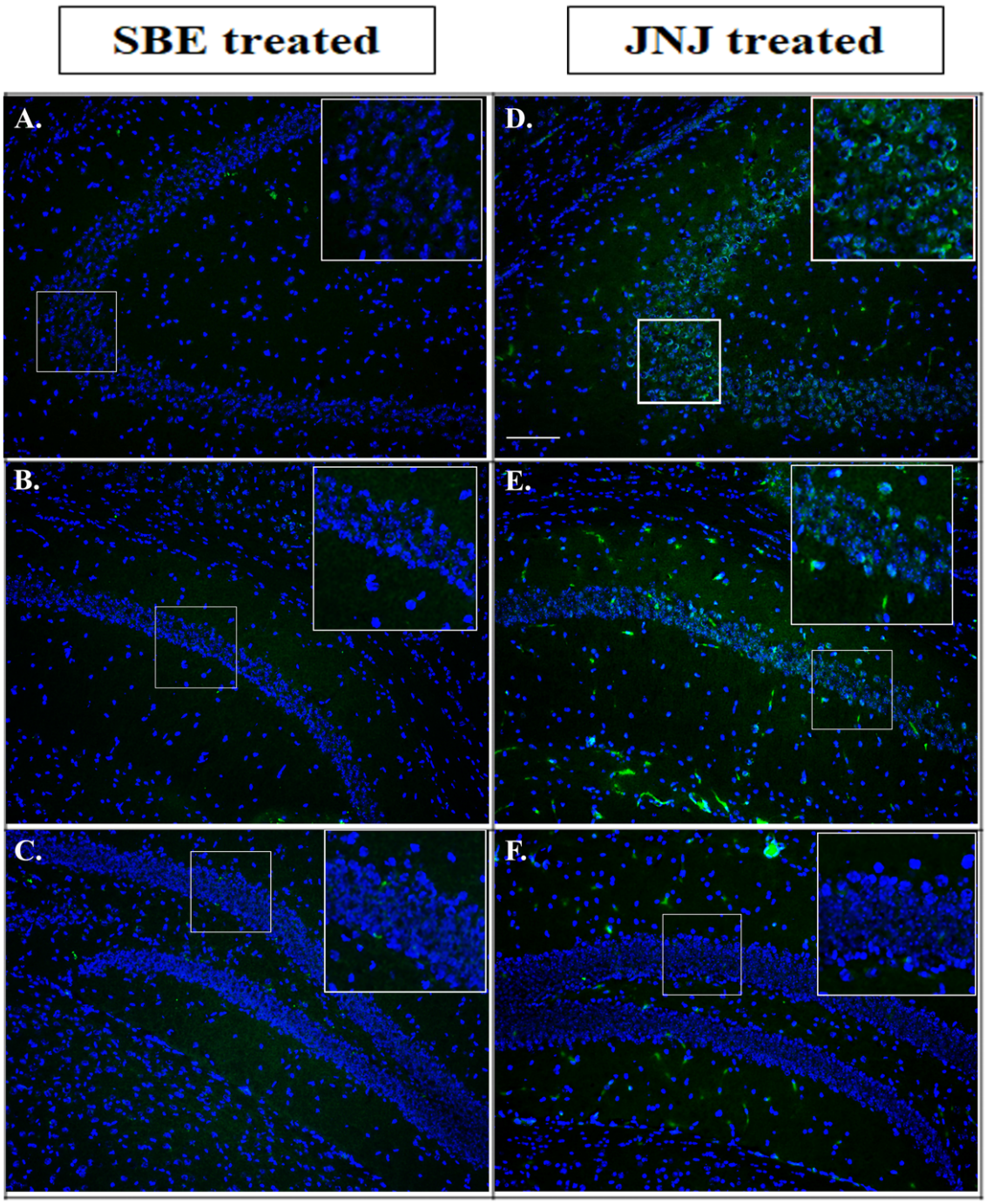


Fig. 3.1. IL-1β immunoreactivity is increased in hippocampal pyramidal neurons after JNJ treatment.

Cohorts of CD-1 mice were treated with 30% SBE-β-CD **(A, B, C)** or 30 mg/kg JNJ-47965567 **(D, E, F)** for 1 hour and brains were removed and processed for immunofluorescence microscopy as described in Materials and Methods. Coronal brain sections were stained for IL-1β (green) and counterstained with DAPI (blue). Representative photomicrographs are shown from the CA3 (A, D), CA1 (B, E) and DG (C, F) sub-regions of the hippocampal formation. Scale bar = 100 μm.

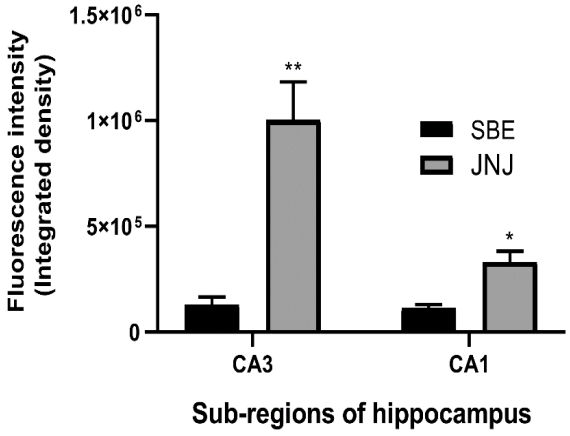


Fig.3.2. Quantification of IL-1β immunoreactivity in the hippocampus.

Immunofluorescence intensity in duplicate sections from 3 vehicle- and 4 JNJ-47965567-treated mice was quantified as described in Materials and Methods. A 2-way ANOVA followed by Tukey's multiple comparisons test was performed on log transformed data. a, significantly different from respective vehicle-treated controls (*, $p = 0.006$); b, significantly different from the corresponding value in the CA3 subregion (*, $p = 0.013$).

3.4.2 Effect of P2X7 antagonist on IL-1β release from hippocampal neurons

IL-1β release was assessed further in cultures of hippocampal neurons. In contrast to the analysis *in vivo* (Fig. 3.3), IL-1β immunoreactivity was detected under basal conditions in the soma of a subset of hippocampal neurons in culture (Fig. 3.3 A), where it appeared as distinct perinuclear puncta. Alongside, it was also expressed in the neuronal processes. This was elevated 30 min after exposure to 0.1 or 0.3 μM JNJ (Fig. 3.3 B and C, respectively).

Immunoblot analysis of hippocampal neuronal cell lysates showed similar elevation in a 31kD protein with increase in pro-IL-1β (Fig. 3.4 A & B). However, there was no evidence for the 17kD processed IL-1β in the cell lysates (Fig. 3.4 C).

Studies herein indicated, both in mice and in cultured hippocampal neurons, P2X7R moderated constitutive IL-1β release, which when blocked pharmacologically, caused IL-1β accumulation in hippocampal neurons.

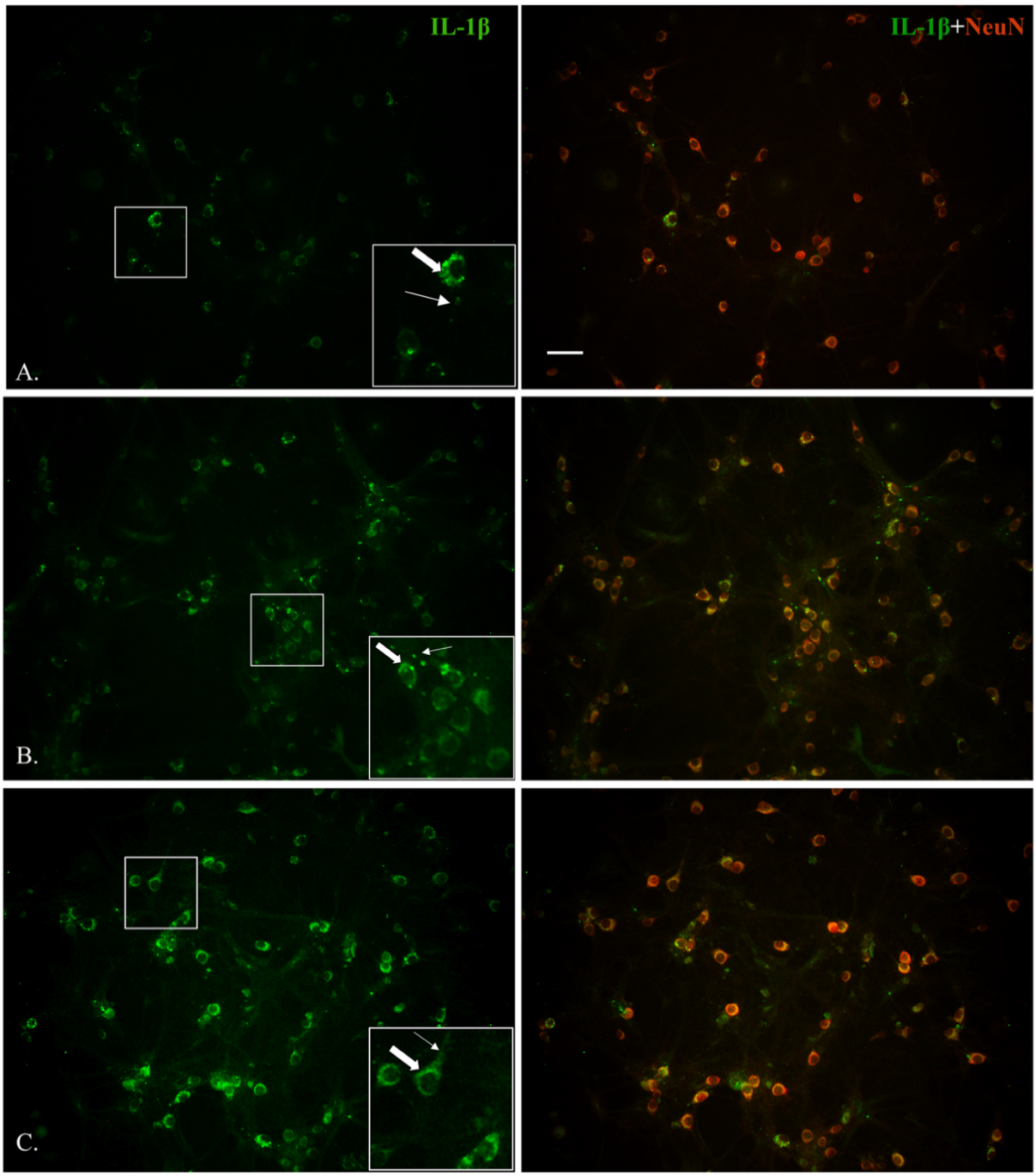


Fig.3.3. IL-1β immunoreactivity is increased in cultures of hippocampal neurons treated with JNJ.

Hippocampal neuron cultures (DIV 14) were treated with DMSO (**A**), 0.1 μM JNJ (**B**), or 0.3 μM JNJ (**C**) for 30 minutes and processed for ICC using anti-IL-1β and without (green, left panel) and with anti-NeuN (red, merged on right panel) as described in Materials and Methods. Scale bar = 25μm. Thick white arrow within inset identifies IL-1β expression in cell body and thin white arrow within inset indicates its expression in the neuronal processes.

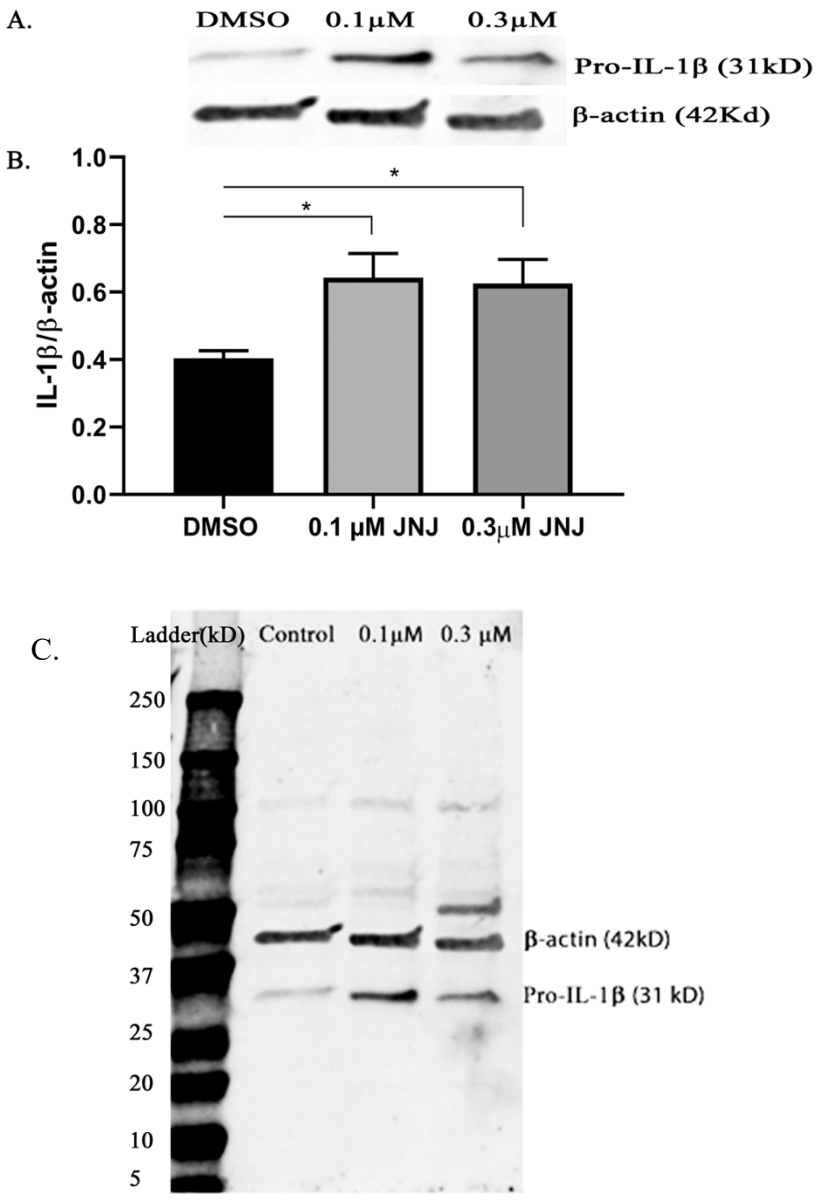


Fig.3.4. JNJ treatment cause an increase in a 31kDa protein in cultures of hippocampal neurons.

Immunoblot analysis using anti-IL-1β and anti-β-actin antibodies was performed on cell lysates harvested 30 minutes following treatment with vehicle (DMSO) or JNJ (0.1 or 0.3 μM) as described in Materials and Methods.

A. Representative immunoblot (**Fig. 3.4 C** shows complete blot). **B.** Quantification of immunoblot results. Densitometric values for the 31kDa protein were normalized to the corresponding 42 kDa β-actin band. Results were analyzed by ordinary one-way ANOVA (*, $p = 0.0373$) followed by uncorrected Fisher's LSD multiple comparisons test. *, indicates significant difference between DMSO vs. 0.1μM JNJ ($p = 0.0209$) and DMSO vs. 0.3μM JNJ ($p = 0.0294$). ($N = 4$).

3.4.3 Localization of IL-1β protein expression in cell bodies and neurites of hippocampal neurons

The subcellular localization of IL-1β in cultures of hippocampal neurons was examined further by higher resolution imaging. Two separate studies (either co-stained with MAP-2 or with NeuN) (Fig. 3.5 and Fig.3.6 respectively) indicated significant elevated IL-1β immunoreactivity in the cell body of neurons with either 0.1μM or 0.3μM JNJ treatment. Under higher resolution, IL-1β expression was punctate and perinuclear in distribution. Although, there was a significant increase in IL-1β immunoreactivity with P2X7R antagonist treatment, there was no change in gross subcellular localization pattern of IL-1β.

IL-1β was also detected in the neuronal processes (Fig 3.7). High resolution images indicated elevated level of IL-1β in neuronal processes proximal to the cell body, which tapered distally along the length of the neurite in both control- and JNJ-treated cells. Quantitation of fluorescent intensity showed that neurons treated with 0.3μM JNJ had significantly elevated IL-1β immunoreactivity in processes compared to control or 0.1μM JNJ-treated cells.

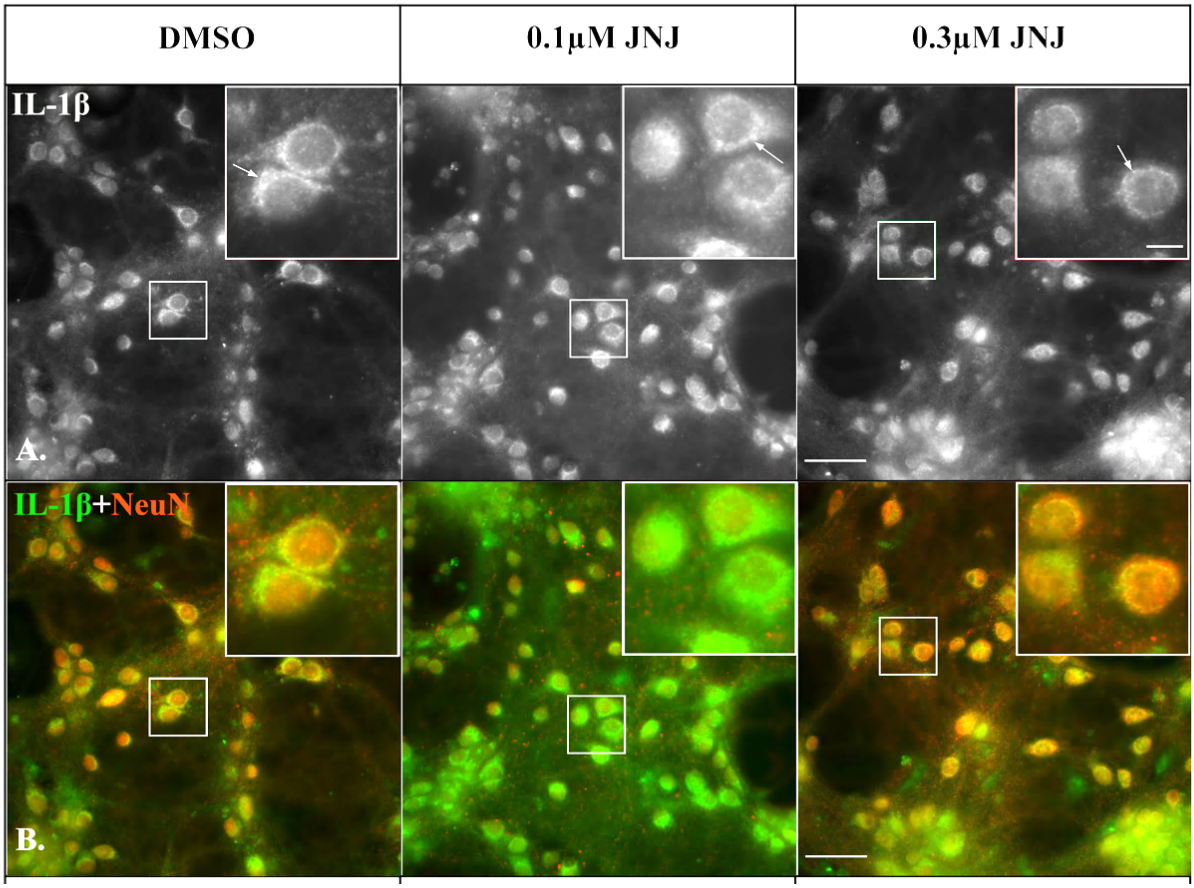
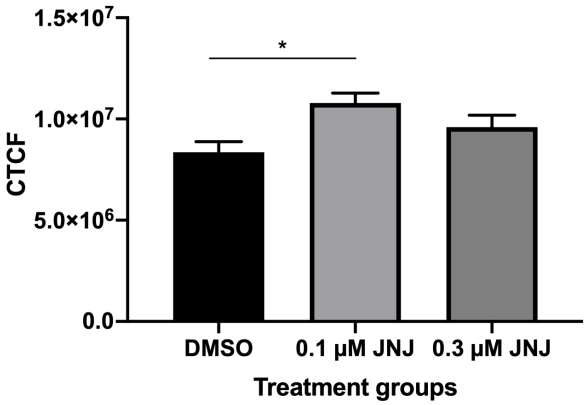


Fig.3.5. Subcellular localization of IL-1β immunoreactivity in cell bodies of cultured hippocampal neurons.

i. Cultures were processed and imaged using high resolution microscopy as described in Materials and Methods. Headings indicate treatments for each column of images. Top panel (A) show respective gray scale images of anti-IL-1β and bottom panel (B) show cultures stained with anti-IL-1β (green) and NeuN (red) antibodies. Scale bar = 25 µm. Images from insets demarcated are in top right corner of respective images. Scale bar = 1µm. Arrows indicate cell bodies stained with anti-IL-1β antibody.



ii. Quantification of IL-1β in neuronal cell body.

Total corrected cell fluorescent intensity of IL-1β measured (as described in methods section) is elevated with 0.1µM or 0.3µM P_2X_7R antagonist treatment in neurons as determined by ordinary one-way ANOVA(*, p=0.0071) followed by uncorrected Fisher's LSD test for multiple comparison (*, p= 0.0017, DMSO vs. 0.1µM JNJ and p= 0.102, DMSO vs. 0.3µM JNJ).

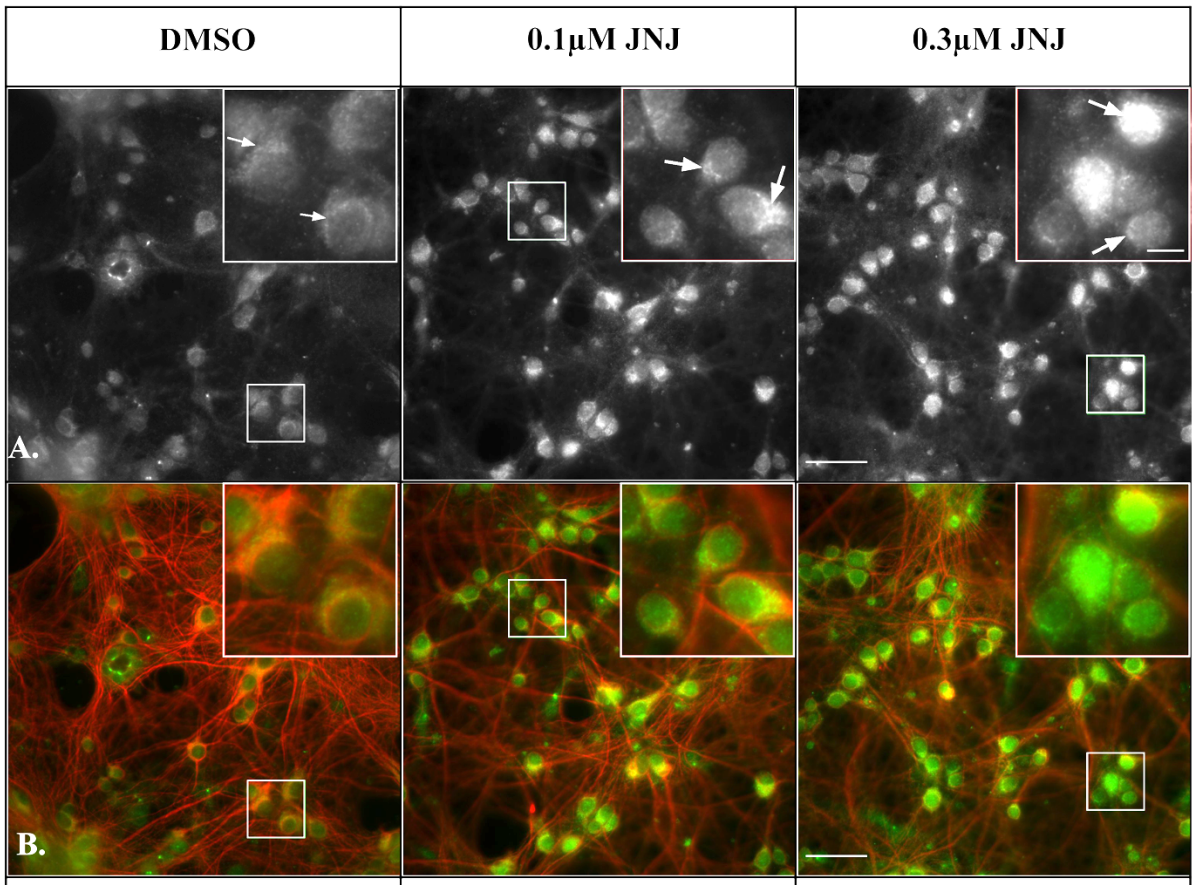
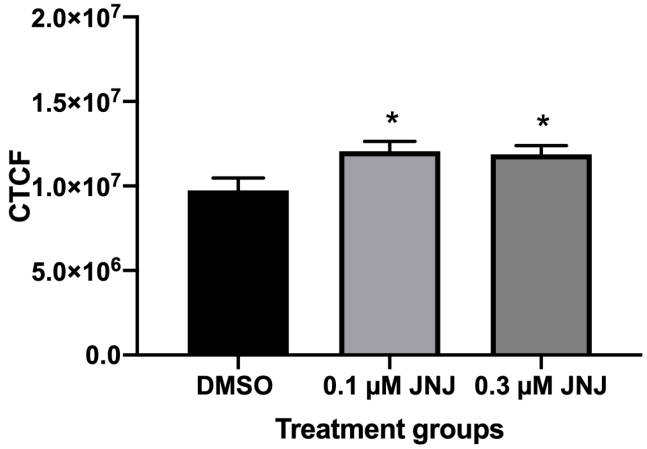


Fig.3.6. Subcellular localization of IL-1β immunoreactivity in cell bodies of cultured hippocampal neurons.

i. Cultures were processed and imaged using high resolution microscopy as described in Materials and Methods. Headings indicate treatments for each column of images. Top panel (A) show respective gray scale images of anti-IL-1β and bottom panel (B) show cultures stained with anti-IL-1β (green) and anti-MAP-2 (red) antibodies. Scale bar = 25 µm. Images from insets demarcated are in top right corner of respective images. Scale bar = 1µm. Arrows indicate cell bodies stained with anti-IL-1β antibody.



ii. Quantification of IL-1β in neuronal cell body.

Total corrected cell fluorescent intensity of IL-1β measured (as described in methods) is elevated with 0.1µM or 0.3µM P_2X_7R antagonist treatment in neurons as determined by ordinary one-way ANOVA (*, p=0.0154) followed by uncorrected Fisher's LSD test for multiple comparison (*, p= 0.0093, DMSO vs. 0.1µM JNJ and *, p= 0.0161, DMSO vs. 0.3µM JNJ).

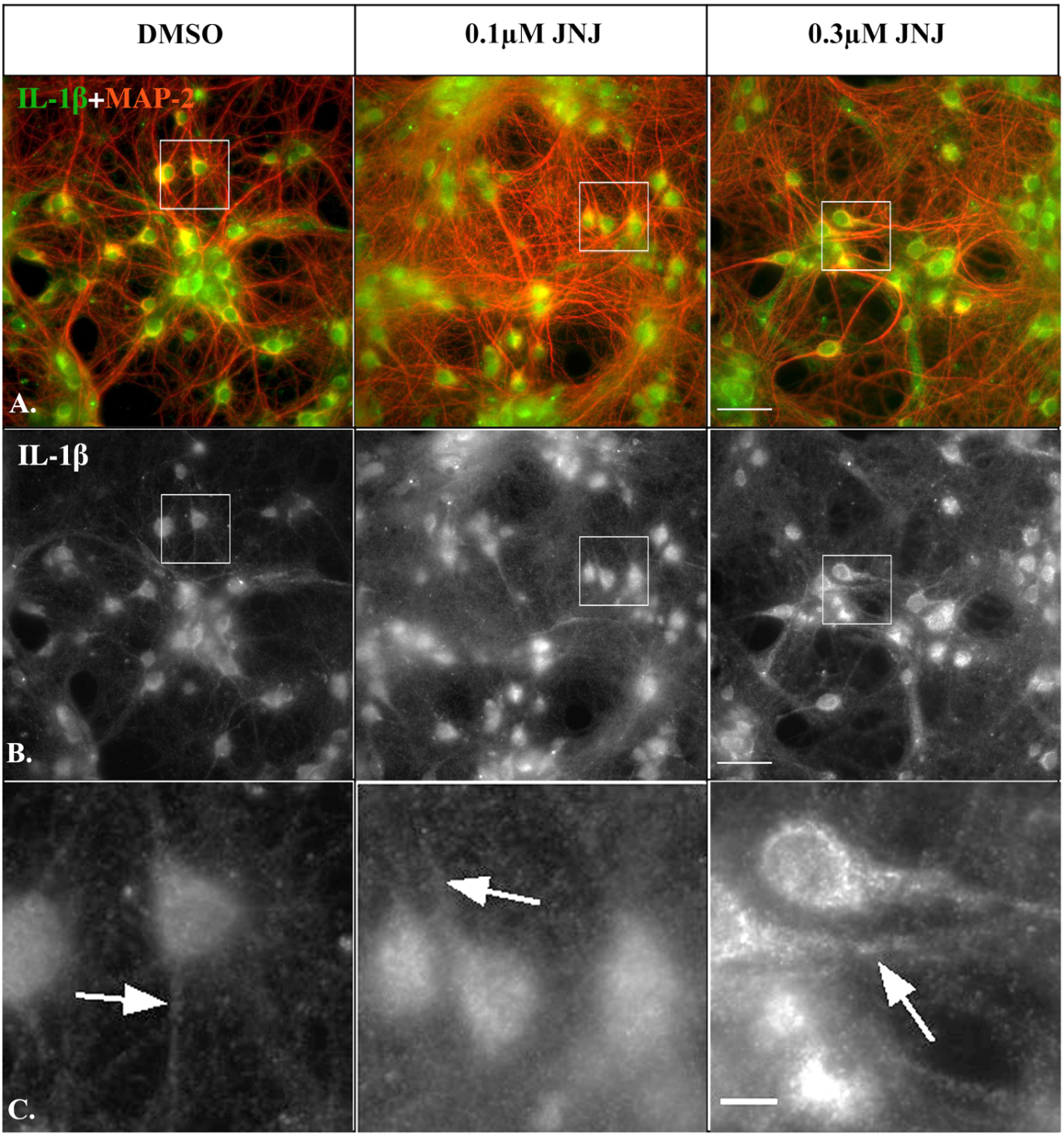
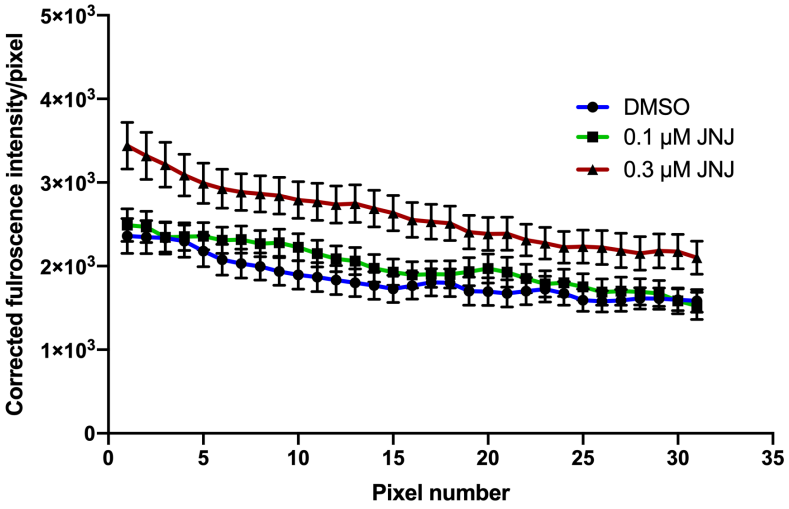


Fig.3.7. Subcellular localization of IL-1β immunoreactivity in neuronal processes of cultured hippocampal neurons.

i. Cultures were processed and imaged using high resolution microscopy as described in Materials and Methods. Headings indicate treatments for each column of images. Top panels (A) show cultures stained with anti-IL-1β (green) and anti-MAP-2 (red) antibodies. Middle panels (B) show respective gray scale images of anti-IL-1β staining from A. Scale bar = 25 µm. Bottom panels (C) are images from insets demarcated in respective panels in B. Scale bar = 1µm. Arrows indicate processes stained with anti-IL-1β antibody.



ii. Fluorescence intensity/pixel of IL-1β in neuronal processes is significantly higher in 0.3 μM JNJ treatment compared to 0.1μM JNJ or vehicle treatment. Immunofluorescence intensity was quantitated as described in Materials and Methods. Significant differences were analyzed using 2-way ANOVA followed by uncorrected Fisher's multiple comparisons test (individual pixel p values in appendix, table 7.1).

3.4.4 Effect of P2X7 antagonist on cFos and COX-2 gene expression

The reduction in seizure threshold observed in mice lacking IL-1 signaling suggests that IL-1 may dampen excitatory transmission in the normal brain [Fig. 1and (Claycomb, Hewett, and Hewett 2012)]. This possibility was assessed in cultures of hippocampal neurons using the immediate early genes, Fos (cFos proto-oncogene) and Ptgs2 (COX-2), as surrogate markers of excitatory neuronal activity (Morgan et al. 1987; Yamagata et al. 1993). First, to demonstrate the relationship between expression and neuronal excitation, cultures of hippocampal neurons were treated with the $GABA_A$ receptor antagonist, bicuculline (Fig 3.8). Compared to vehicle-treated cultures, bicuculline induced a 7-fold increase in cFos mRNA expression and a 6-fold elevation in COX-2 mRNA expression (Fig. 3.8, panels A and B, respectively). However, there was a lack of effect of NMDA receptor antagonist, APV, which is in agreement to what is expected, as APV treatment suppresses expression mediated by excitatory activity (Fig. 3.8, panels A and B, respectively).Next, the effect of JNJ on cFos and COX-2 expression was assessed (Fig. 3.8, panels C and D, respectively). Although more modest than bicuculline, treatment with 0.1 and

0.3 μM JNJ enhanced cFos expression by 1.86 and 2.16 fold and COX-2 mRNA expression by 1.68 and 1.81-fold, respectively.

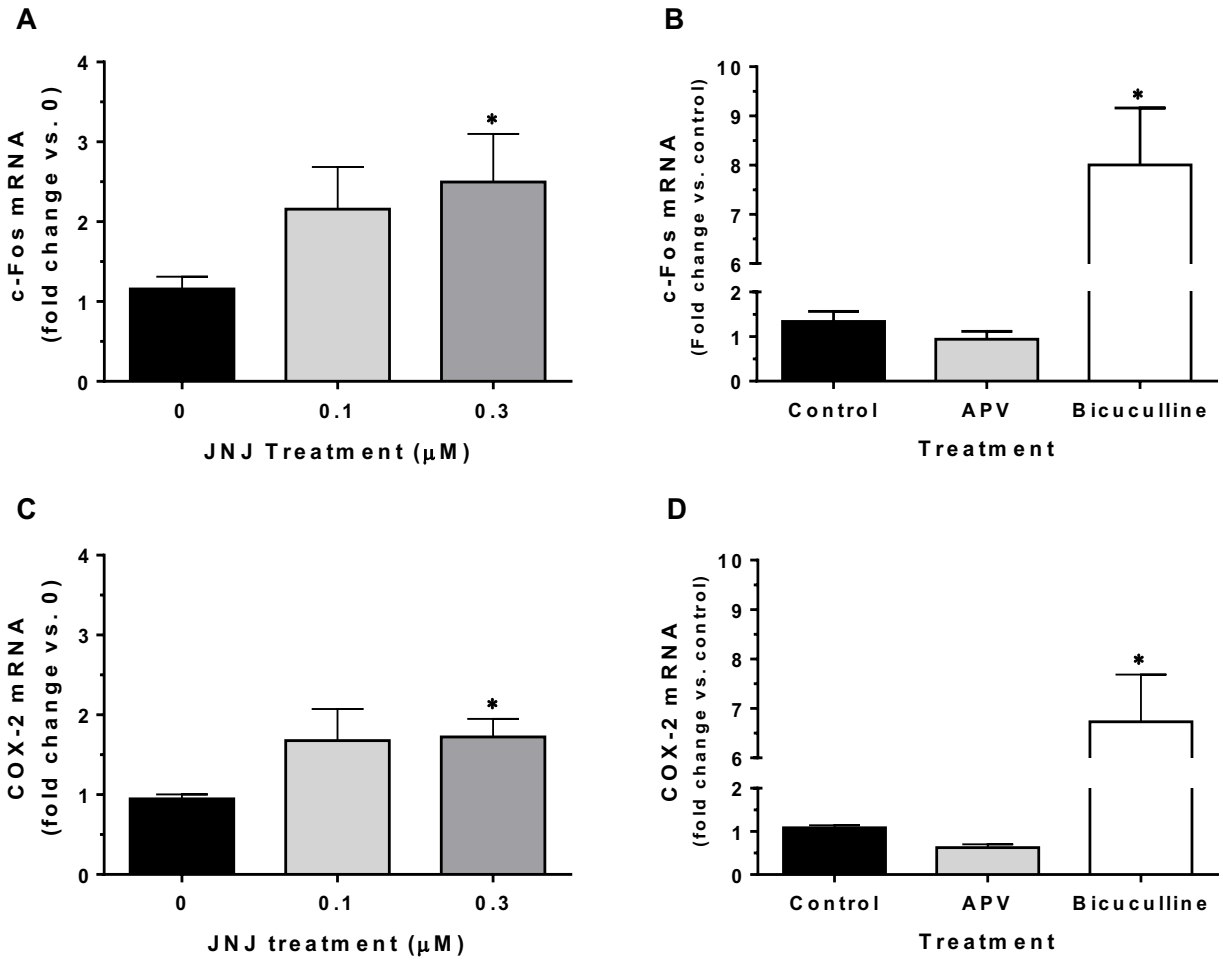


Fig.3.8. cFos and COX-2 mRNA expression are increased in cultures of hippocampal neurons after JNJ.

cFos (**A**, **B**) and COX-2 (**C**, **D**) mRNA expression was assessed by quantitative PCR analysis as described in Materials and Methods. Cultures were exposed to 0 (DMSO), 0.1μM, or 0.3 μM JNJ for 30 minutes (**A**, **C**) and 30mM APV or 100 mM bicuculline for 90 minutes (**B**, **D**).

A. c-Fos mRNA expression significantly increased with 0.3μM JNJ treatment (N=4) however, not with 0.1M JNJ (N=4) as determined by Kruskal Wallis test (*, p= 0.0002) followed by uncorrected Dunn's test for multiple comparisons (*, p= 0.0017, DMSO vs. 0.3μM JNJ and p= 0.1160, DMSO vs. 0.1μM JNJ).

(continued)

B. COX-2 mRNA expression significantly increased with 0.3 μM JNJ treatment (N=4) however, not with 0.1μM JNJ treatment (N=4) as determined by Kruskal Wallis test (p= 0.0876) followed by uncorrected Dunn's test for multiple comparisons (*, p= 0.0307, DMSO vs. 0.3μM JNJ and p= 0.4320, DMSO vs. 0.1μM JNJ).

C. c-Fos mRNA expression significantly increased in hippocampal neuronal cells with 100μM bicuculline treatment (N=4) but not with 30μM APV treatment (N=4) compared to control (N=4), as determined by ordinary One-way ANOVA (*, *p*=<0.0001) followed by uncorrected Fisher's LSD test for multiple comparisons (*, *p*=<0.0001, control vs. Bicuculline and *p*= 0.6904, control vs. APV).

D. COX-2 mRNA expression significantly increased in hippocampal neuronal with 100μM bicuculline treatment (N=4) but not with 30μM APV treatment (N=4) compared to control (N=4), as determined by ordinary One-way ANOVA (*, p=<0.0001) followed by uncorrected Fisher's LSD test for multiple comparisons (*, p=<0.0001, control vs. Bicuculline and p= 0.5717, control vs. APV).

3.4.5 Effect of P2X7 antagonist on PTZ-induced seizure activity

The effect of JNJ on basal IL-1β release together with its effect on cFos and COX-2 expression (Fig.3.8) further suggest that constitutive IL-1β release serves to dampen excitatory neurotransmission in the normal hippocampus. These results raise the possibility that JNJ will enhance the sensitivity to PTZ, similar to that observed in IL-1R1 deficient mice (results from chapter 2). To test this hypothesis, PTZ-induced seizure responses were assessed 60 min following treatment with JNJ or its vehicle, SBE (Fig. 3.9). Seizure severity was significantly increased in JNJ-treated mice relative to mice treated in parallel with the SBE vehicle (Fig. 3.9A). Although the incidence of convulsive seizures was increased two-fold in the JNJ treatment group vs. the SBE control group ($\chi 2$ test for linear trend, $p = < 0.0001$), neither this nor the latency to convulsions were significantly changed (Fig. 3.9 B and C). The effect on convulsive seizure incidence was robust as it was also observed with higher doses of PTZ (Fig.3.10 and 3.11). There was a dose-dependent increase in convulsive seizures with 46 and 50 mg/kg PTZ in the SBE-treated mice (40% and 50%, respectively). This nearly paralleled by correspondingly higher incidences in the JNJ-treated mice (70 and 75% for 46 and 50 mg/kg PTZ, respectively).

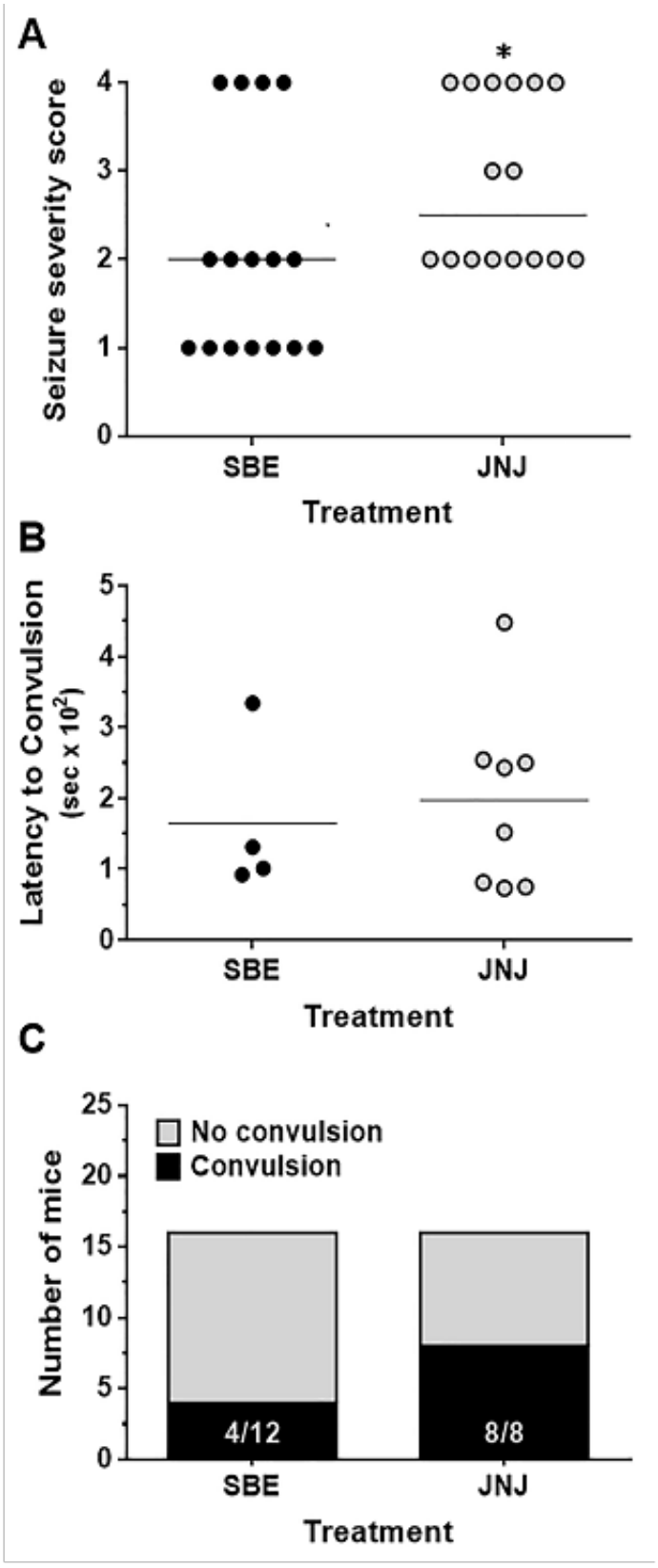


Fig.3.9. P2X7R antagonist lowers seizure threshold.

Acute seizure activity was induced by PTZ (42 mg/kg, i.p.) in vehicle and JNJ treated mice and seizure behavior was scored on a 5-point scale of increasing severity as described in methods.

A. Seizure severity. Each point represents the maximum seizure score for an individual mouse. The median seizure scores for between treatments (horizontal line) are statistically different (*, p=0.0219, Mann Whitney test, two tailed).

B. Latency to convulsion. There is no significant difference between the treatments (p =>0.999, two-tailed Mann-Whitney test). Horizontal line indicates mean values.

C. Incidence of convulsions. The number of mice exhibiting a convulsive seizure (PTZ seizure score≥3) in A is expressed as a fraction of total mice injected with PTZ for each treatment group (ratio in the bar is the raw data. (χ2 test for linear trend, *p= < 0.0001).

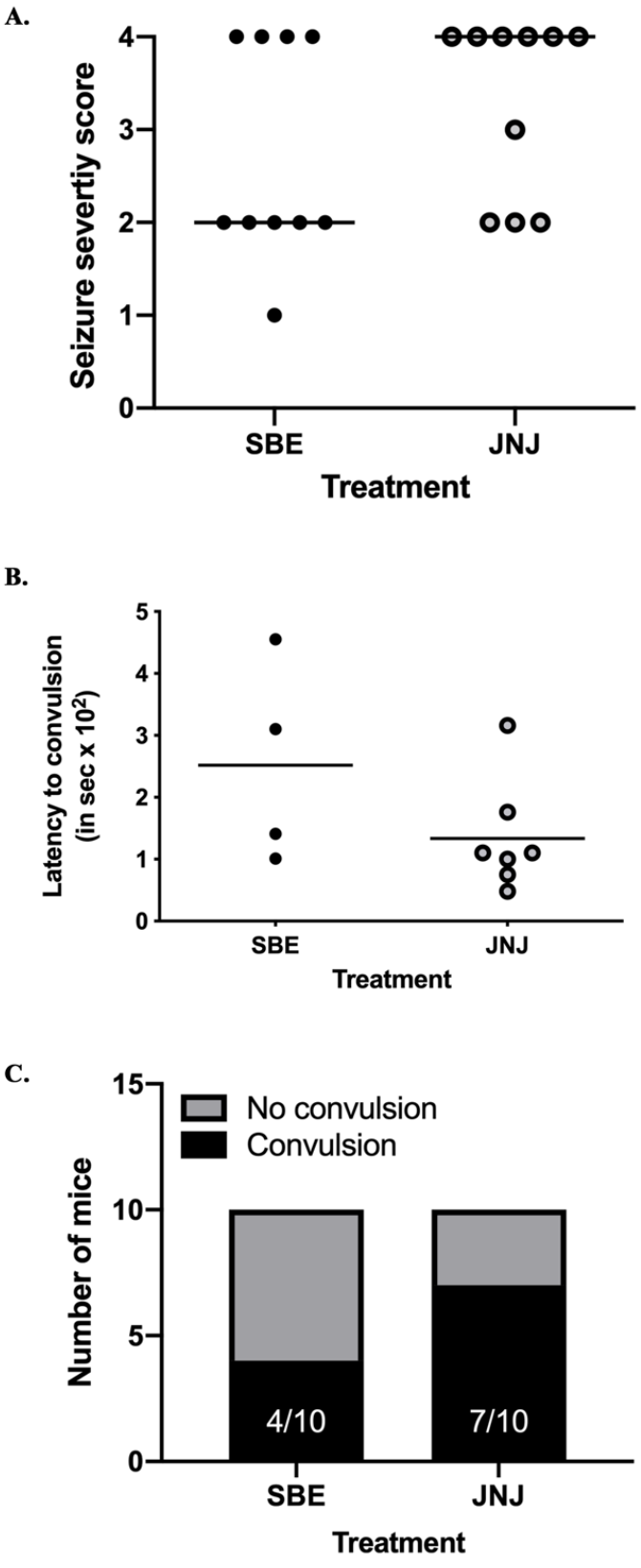


Fig.3.10. P2X7R antagonist lowers seizure threshold.

A. For 46 mg/kg PTZ, seizure severity. Each point represents the maximum seizure score for an individual mouse. The median seizure scores between treatments (horizontal line) are not statistically different (p=0.306, Mann Whitney test, two tailed).

B. Latency to convulsion. There is no significant difference between the treatments (p=0.2121, two-tailed Mann-Whitney test). Horizontal line indicates mean values.

C. Incidence of convulsions. The number of mice exhibiting a convulsive seizure (PTZ seizure score≥3) in A is expressed as a fraction of total mice injected with PTZ for each treatment group (ratio in the bar is the raw data). ($\chi 2$ test for linear trend, *p= < 0.0001).

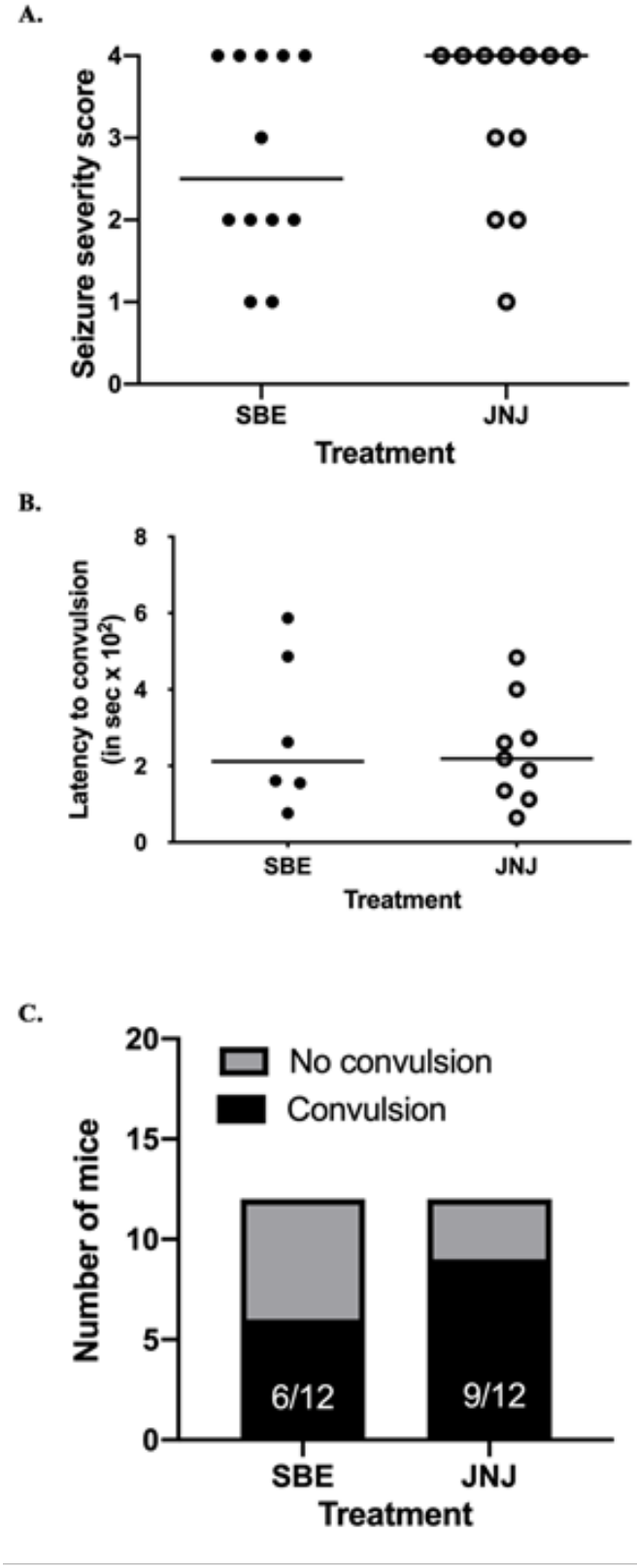


Fig.3.11. P2X7R antagonist lowers seizure threshold.

A. For 50 mg/kg PTZ, seizure severity. Each point represents the maximum seizure score for an individual mouse. The median seizure scores between treatments (horizontal line) are not statistically different (p=0.3622, Mann Whitney test, two tailed).).

B. Latency to convulsion. There is no significant difference between the treatments (p=0.6889, two-tailed Mann-Whitney test). Horizontal line indicates mean values.

C. Incidence of convulsions. The number of mice exhibiting a convulsive seizure (PTZ seizure score≥3) in A is expressed as a fraction of total mice injected with PTZ for each treatment group (ratio in the bar is the raw data). ($\chi 2$ test for linear trend, *p= 0.0141).

3.5 Discussion

Evidence from this study supports three primary suppositions. First, pyramidal neurons, but not granule cells of the dentate gyrus, are the primary cellular source of physiological extracellular IL-1β release in the normal hippocampus. Second, basal processing and release of IL-1β from these neurons is dependent on P2X7R activity. Third, P2X7R contributes to the homeostatic balance of excitation and inhibition in the brain.

3.5.1 Cellular source of constitutive IL-1β release in the normal hippocampal formation

Several lines of evidence from previous studies support the contention that IL-1β expression and release occur under physiological conditions in the brain. First, IL-1β transcripts have been detected under basal conditions in RNA isolates from several tissues, including the hypothalamus and hippocampus (Quan et al. 1996; Hosoi, Okuma, and Nomura 2000a), and this was shown to fluctuate on a diurnal schedule (Taishi et al. 1997). Second, picomolar levels of IL-1β protein have been reported in tissue homogenates from these regions indicating that the cytokine is translated (Watt and Hobbs 2000; Hosoi, Okuma, and Nomura 2000b; Takemiya et al. 2017). Third, tissue dialysis measurements suggest that IL-1β protein is released constitutively into the extracellular space *in vivo* (Phelps et al. 1995; Summy-Long et al. 2008; Frank et al. 2020) and immunohistochemical results showing physiological release of IL-1β from the neurohypophysis is consistent with this (Watt and Hobbs 2000). Additionally, low picomolar levels of IL-1β protein were detected in supernatants from hippocampal slice cultures under basal conditions and this release occurred at 37 but not 20^{o}C, demonstrating that IL-1β protein secretion required normal cellular metabolic processes (Ross et al. 2003).

The identity of the cellular sources of IL-1β release is key to understanding its signaling in the normal brain. In the hypothalamus, IL-1β protein expression has been localized to oxytocin- and

vasopressin-containing neurons of the magnocellular and neurohypophysis suggesting a role in regulation of normal physiological processes and behavior (Watt and Hobbs 2000). In the hippocampus, results from one study showed that IL-1β mRNA was expressed in each of the three major glutamatergic neuron populations of the rat hippocampal formation - the DG, CA3, and CA1 (Bandtlow et al. 1990). Another study, however, reported prominent constitutive IL-1β immunoreactivity in the mossy fiber axon terminals originating from the DG, but apparently did not find evidence for expression in pyramidal neurons of the CA3 or CA1 (Lechan et al. 1990). These results are at odds with the current study, which found IL-1β immunoreactivity in the hippocampus only after treatment with the P2X7R antagonist, and under this condition, the immunoreactivity was localized to CA3/CA1 pyramidal neurons but not granule cells of the DG. The reason for the disparate findings between this and the Lechan study is not clear. However, it could be due to species differences in IL-1β expression in the hippocampal formation since the latter study was performed using tissue from rat brains. Possible species differences in IL-1β function in the brain have been discussed previously (Vitkovic et al. 2000).

The cellular target of IL-1β action in the mouse hippocampal formation under basal conditions remains to be elucidated. However, the location of IL-1R1 expression may provide hints. The highest level of IL-1R1 mRNA expression is found in the granule cells of the DG and this expression profile correlates well with the location of ligand binding sites, which are highly concentrated in these cells as well (Ban et al. 1991; Cunningham et al. 1992; Deyerle et al. 1992). Given the evidence herein implicating CA3 neurons as a major source of IL-1β in the hippocampus, this expression profile raises the possibility that CA3 may modulate DG function via a pathway involving IL-1β-IL-1R1 signaling. A recent study using a novel transgenic approach to map IL-1R1 protein expression in the hippocampal formation found prominent IL-

1R1 protein in the cell soma and molecular layer of DG granule cells (Liu et al. 2015). The latter is consistent with another study showing post-synaptic localization of IL-1R1 in hippocampal neurons (Gardoni et al. 2011). Expression was also detected in the mossy fiber tract of the DG neurons, suggesting a possible presynaptic function of IL-1 signaling as well.

3.5.2 Role of P2X7R in constitutive IL-1β release in the normal hippocampus

The detection of IL-1β immunoreactivity in hippocampal pyramidal neurons in the presence but not absence of the P2X7R antagonist is consistent with the following conclusions: 1) IL-1β is constitutively released by these neurons at very low levels in the normal hippocampus, 2) blocking the function of P2X7R caused the cytokine to accumulate in these cells, and 3) P2X7R functions as a trigger for IL-1β release from these cells under normal conditions *in vivo*. The results from primary hippocampal neuron cultures showing an increase in IL-1β immunoreactivity following treatment with the P2X7R antagonist provide additional support for the role of P2X7R in constitutive release of IL-1β from hippocampal neurons. Evidence showing high P2X7R mRNA expression levels in pyramidal neurons, and in particular CA3 pyramidal neurons, is consistent with these conclusions [(Metzger et al. 2017) and Allen Mouse Brain Atlas]. It should be recognized, however, that not all evidence agrees with this expression pattern. For example, one study reported P2X7R mRNA expression in all primary cells of the rat hippocampal formation, including the DG (Sperlágh et al. 2002). On the other hand, another study reported presynaptic expression of P2X7R protein in the rat mossy fiber terminals of the DG (Armstrong et al. 2002), which is interesting given the reported location of IL-1β ligand in these same termini (Lechan et al. 1990). This draws further attention to the possibility that species differences in IL-1β signaling could exist in the normal hippocampus. Alternatively,

since the approach by Metzger et al. eliminated all splice variants of P2X7R, it is possible that some differences in express patterns between studies may be due to splice variants.

It is intriguing that the immunostaining in hippocampal neuron cultures appeared most prominently as perinuclear puncta. The nature and function of these puncta will require further investigation. However, one possibility is that they are micro-vesicles that transport IL-1β from the cell soma to remote sites of release, similar to what has been proposed in the hypothalamus (Watt and Hobbs, 2000). The detection of small puncta in the proximal region of neurites herein is consistent with this possibility. It is important to note that IL-1β release does not follow the classical endoplasmic reticulum-Golgi apparatus secretory pathway (Rubartelli et al. 1990) and that several alternative vesicle-mediated mechanisms have been proposed for its release from activated inflammatory cells (Dubyak 2012). Among these pathways are microvesicle shedding (Monteleone et al. 2018), secretory lysosome release (Rubartelli et al. 1990; Andrei et al. 1999) or exosome secretion (Qu et al. 2007). Qu et al. argued that the exosome pathway is the primary release mechanism from inflammatory cells. However, in addition to the puncta, a second fraction of diffuse cytosolic IL-1β immunoreactivity was observed in cell soma and proximal neurites of hippocampal neurons in culture. A non-vesicular fraction of IL-1β was also reported in activated monocytes (Rubartelli et al. 1990; Andrei et al. 1999). The significance of this fraction is not clear, although it would be consistent with release via micro-vesicle shedding (Muralidharan-Chari et al. 2010).

Since the antibody used in immunocytochemical analysis herein detects both pro and active IL-1β, it was not possible to determine whether the immunoreactivity was due to unprocessed/pro or processed/active IL-1β. However, the results from the IL-1β immunoblot analysis may be informative. The detection of an ~31-kDa band and absence of a 17kDa band in the culture

lysates suggests that the immunoreactivity is due to unprocessed (pro) IL-1β and that the active form is not stored but released upon processing. Additionally, the conspicuous absence of a 17kDa band and small increase the 31kDa band in cultures treated with the P2X7R antagonist further supports this notion and implies that the increase in immunoreactivity in these cultures was due to accumulation of the unprocessed peptide. An important implication of this result is that processing as well as release of IL-1β occur in a P2X7R-dependent manner similar to what has been reported in inflammatory cells (Piccini et al. 2008). The primary difference is that the neuron cultures were not exposed to inflammatory stimuli. How IL-1β is process under non-inflammatory conditions remains an open question. It is interesting to note that in addition to expression of caspase-1, primary cultures of neurons from human embryonic brains constitutively express several components of inflammasomes under normal conditions, raising the possibility that neuronal IL-1β may be processed by an inflammasome-like process (Kaushal et al. 2015). However, it is not known whether these factors are expressed in the normal adult hippocampus. Alternatively, it is possible that processing occurs in an entirely novel manner. In this regard, caspase-1 dimerization has been shown to be sufficient for IL-1β processing in the absence of an inflammasome (Conos et al. 2016). In any case, IL-1β is presumed to be processed by caspase-1 and there is evidence to support this in the hypothalamus (Tringali et al. 1996). In this regard, a preliminary study performed with Caspase-1 inhibitor, Y-VAD-CHO for 1 hour, showed a dose dependent, mild increase in pro-IL-1β level in hippocampal neurons with western blot analysis (study described in appendix, Fig.7.8). Although further research needs to be conducted to confirm role of Caspase-1 in cleavage and release of processed IL-1β in hippocampal neurons, this data may indicate a role of Caspase-1 in this process.

3.5.3 Role of P2X7R in modulation of the excitatory-inhibitory (E/I) balance

Two approaches were used to examine the role of constitutive P2X7R in maintenance of the homeostatic balance between excitation and inhibition under physiological conditions. The first examined the effect of P2X7R antagonist on the activity of the Fos and Ptgs2 genes in cultures of hippocampal neurons. Because the transcriptional activity of these immediate early genes, which code for cFos and COX-2, is coupled to NMDA receptor signal transduction (Morgan et al. 1987; Cole et al. 1989; Yamagata et al. 1993; Hewett et al. 2016), the increase in expression of their mRNA expression in neurons after exposure to the antagonist is consistent with a modulatory role of P2X7R on excitatory-inhibitory homeostasis in the normal hippocampus. Specifically, the result suggests that constitutive P2X7R activity, likely driven by constitutive ATP release, dampens excitation in the hippocampus under basal conditions. While the increase is small, it is important to note that it occurred in the context of strong basal $GABA_A$ receptor-mediated inhibition in hippocampal neuron cultures, as indicated by the marked increase in cFos and COX-2 expression induced by bicuculline in the neuronal cultures. On the other hand, the lack of effect of APV by itself, which would be expected to suppress expression mediated by excitatory activity, indicates that this basal inhibitory tone was sufficient to suppress to a large extent excitatory activity in these cultures.

The second approach to assess the function of P2X7R in maintenance of E/I homeostasis examined the effect of the P2X7R antagonist on the PTZ-induced convulsive seizure threshold. As a $GABA_A$ receptor antagonist (Ramanjaneyulu and Ticku 1984; Huang et al. 2001), PTZ shifts the homeostatic balance between excitation and inhibition in the brain to favor excitation. This effect is dose-dependent and, if disinhibition is sufficiently strong, it can disrupt normal brain function, triggering a seizure response. Results herein demonstrating that the P2X7R

antagonist enhanced sensitive to the excitatory properties of PTZ further suggests that constitutive activity of P2X7R is involved in maintenance of the E/I balance in the normal brain.

Considering the effect of the antagonist on IL-1β release in the hippocampus, the result from these studies are consistent with the hypothesis that the effect of the antagonist on cFos/COX-2 expression in neuronal cultures and PTZ seizure threshold *in vivo* is due to blocking IL-1β release in the hippocampus. However, it is important to acknowledge that the effect of the antagonist on IL-1β release and E/I balance are correlative, and as such, results herein cannot directly address cause and effect. By virtue of its properties as a non-selective cation channel, P2X7R could influence neuronal function independent of a role in IL-1β release. Moreover, constitutive IL-1β expression has been reported in brain regions in addition to the hippocampus, including the cortex, that could influence E/I balance. Thus, a relationship between P2X7R and hippocampal IL-1β release in maintenance of E/I balance awaits more direct evidence. Clearly, much remains unknown about the biochemistry of IL-1β in the normal hippocampus and additional studies are necessary to answer questions related to its production, release, and signaling. However, results from the current study will provide a foundation for further investigation.

Specific Aim 3: ***To investigate the role of excitatory neuronal activity on IL-1β expression/ signaling function in neurons of the hippocampus: possible link between IL-1β signaling and Cyclooxygenase-2 (COX-2)***

Synopsis

Aim 3 analyzed the possibility of whether alteration in expression of endogenous IL-1β ligand or it's signaling components expression and/or its signaling functionalities downstream may occur due to excitatory neuronal activity. This aim parallelly investigated two sub-aims:

1. IL-1β release was found to be activity-dependent in the hypothalamus (Watt and Hobbs 2000) or in hippocampal slice cultures (Ross et al. 2003), however, this has not been thoroughly examined in the lights of seizure activity which is an outcome of uncontrolled neuronal activity. These aims hypothesized IL-1β expression may be dependent on excitatory neuronal activity and this idea would be explored in the first part of this chapter.

2. COX-2 has neuromodulatory role in maintenance of the innate seizure threshold (Claycomb, Hewett, and Hewett 2012; Gong and Hewett 2018). Of importance to the present study, exogenous IL-1β had been shown to induce COX-2 expression and prostaglandin production in different CNS cells types (Samad et al. 2001; Sayyah et al. 2005; Neeb et al. 2011) suggesting neuromodulatory pathway of COX-2 may function downstream of IL-1β. Furthermore, previous study from our lab showed COX-2 inhibition elevated seizure severity in mice similarly in wildtype mice and in mice with genetic deletion of Il-1R1. These studies together suggested a possible contribution of COX-2 functioning downstream of IL-1β signaling, and this would be explored in the second part of this chapter. Taken together, these two studies would investigate effects of neuronal excitation on endogenous IL-1β expression and on its downstream signaling possibly via COX-2.

Chapter 4

Effect of neuronal excitation on endogenous IL-1β:

Possible contribution of COX-2

4.1 Summary

Inactivating Interleukin-1β (IL-1β) or its signaling receptor (IL-1R1), genetically, or IL-1β release from neurons, pharmacologically, sensitized mice to the convulsant property of pentylenetetrazol (PTZ), implying its constitutive presence in maintenance the innate seizure threshold. Still, whether changes in excitatory neuronal activity could simultaneously alter IL-1β synthesis and/or downstream signaling function remains to be elucidated. This was examined *in vivo* using PTZ induced convulsion and in hippocampal neuron culture with bicuculline. Effect of convulsion transiently induced IL-1β mRNA *in vivo*. Although, low endogenous IL-1β protein could be detected in mice hippocampus and in cultured neurons, excitatory neuronal activity had no immediate effect on the basal protein level.

Secondly, purpose of this study was also to investigate if neuro-modulatory functions of endogenous IL-1β is mediated via Cyclooxygenase-2 (COX-2). Therefore, this study determined whether endogenous IL-1β affected basal and induced COX-2 expression and/or activity. Mice with genetic deletion of IL-1R1 exhibited lower basal and PTZ induced COX-2 expression in hippocampus. Parallelly, neutralizing IL-1R1 in cultured hippocampal neurons lowered basal and bicuculline induced COX-2 immunoreactivity. As COX-2 is the rate limiting enzyme of prostaglandin synthesis pathway, lower endogenous COX-2 expression in IL1r1 mutant mice hippocampus also paralleled with altered basal PGE_2 profile compared to wildtype littermates and affected convulsion induced PGE_2 production in mutants. This study will have implication in understanding how endogenous IL-1β may maintain homeostatic excitation-inhibition balance via COX-2.

4.2 Introduction

Interleukin-1β (IL-1β) is a well-characterized cytokine of the innate and adaptive immune systems (Dinarello 2009). Within the central nervous system (CNS), IL-1β has been implicated in the pathogenesis of several neuroinflammatory and neurodegenerative diseases (Rothwell and Luheshi 2000; Simi et al. 2007; Fogal and Hewett 2008; Shaftel, Griffin, and O'Banion 2008; Pinteaux, Trotter, and Simi 2009; Hewett, Jackman, and Claycomb 2012). Several exogenous (bacterial products) and endogenous factors (pro-inflammatory cytokines) causes increase in IL-1β level and its release in brain (Rothwell and Luheshi 2000; Gibson, Rothwell, and Le Feuvre 2004; Simi et al. 2007). Parallelly, it's function is also described in specific physiological functions of the CNS independent of its role as immune mediator such as sleep (Krueger et al. 1998; Opp, Obal, and Krueger 1991), body fluid regulation (Diana et al. 1999; Summy-Long et al. 2006; 2008), neuroprotection (Strijbos and Rothwell 1995; Mason et al. 2001), learning and memory (Schneider et al. 1998; Barrientos et al. 2002; Ross et al. 2003; Avital et al. 2003). Very low constitutive expression of IL-1β mRNA and protein have been reported in various regions of the normal brain of rat: hypothalamus (Watt and Hobbs 2000), hippocampus (Lechan et al. 1990; Kaneko et al. 2006; Viviani et al. 2014) and cortex (Quan et al. 1996), indicative of its neuromodulatory functions. IL-1β signals through the canonical plasma membrane receptor complex consisting of the ligand-binding chain, Interleukin-1 receptor 1 (IL-1R1), and its accessory protein, IL-1RacP. Upon ligand activation, MyD88 is recruited to this complex, where it serves as an adaptor for binding of Interleukin receptor-associated kinases (IRAKs) to subsequently initiate downstream signaling cascade (Dinarello 2002; 2009). All components of canonical IL-1 signaling are also expressed in the normal brain at the physiological level (French et al. 1999; Nadjar et al. 2005; Cremona et al. 1998; Andre et al. 2005; Yabuuchi et al. 1994;

Cunningham et al. 1992) and in cultured hippocampal neurons (Gardoni et al. 2011), implying the likelihoods of neuromodulatory functions of constitutive IL-1β within the physiological brain. Particularly to our interest, constitutive IL-1β is expressed in CA3 pyramidal neurons of murine hippocampus (Chapter 3) and that blocking constitutive IL-1β signaling either genetically or its release pharmacologically may lower the seizure threshold in mice [(Claycomb, Hewett, and Hewett 2012) and Chapter 3]. These findings are indicative of neuromodulatory role of constitutive IL-1β in regulating the homeostatic E/I balance. However, it is unclear whether changes in neuronal activity (uncontrolled electrical activity which leads to the acute seizure) could alter IL-1β expression and function. This study will focus on the effect of acute seizure on IL-1β expression in normal hippocampus. Secondly, this study will also try to identify any downstream effector candidate of IL-1β, which may play a role in moderation of E/I balance in an IL-1β dependent manner.

In the present study, an interplay between constitutive IL-1β signaling and one of its known downstream effectors, Cyclooxygenase-2 (COX-2) expression and/or activity in neuromodulation is therefore investigated. Cyclooxygenases (COX) are enzymes that catalyze the rate-limiting step of Arachidonic acid (AA) metabolism, producing lipid immune mediators called prostaglandins (PGs) (Kaufmann et al. 1996). Unlike COX-1, COX-2 is constitutively expressed by certain excitatory neurons of CNS (Förstermann et al. 1982; Yoshikawa et al. 2006). Constitutive COX-2 mRNA expression in cultured cortical neurons is NMDAR dependent (Hewett et al. 2016). In relevance to this study, previous results from our lab indicated overexpression of neuronal COX-2 in mice maintained elevated seizure threshold (Gong and Hewett 2018) and inhibiting COX-2 markedly increased susceptibility of mice to seizure induction (Claycomb, Hewett, and Hewett 2011), suggesting that COX-2 contributes to the

maintenance of the innate seizure threshold. COX-2 is thus widely studied in CNS for its role in several neuronal diseases as well as for its neuromodulatory functions, including seizure and epilepsy (Hewett, Bell, and Hewett 2006).

Interestingly, inflammation mediated or exogenous IL-1β induces COX-2 expression and/or prostaglandin (PG) production in different CNS cell types (Serou, DeCoster, and Bazan 1999; Fiebich et al. 2000; Bazan and Lukiw 2002; Moolwaney and Igwe 2005; Favrais et al. 2007). Of importance, a link between these two pathways has been demonstrated in central sensitization to inflammatory pain *in vivo* (Samad et al. 2001; Sayyah et al. 2005) and in rat trigeminal nerve ganglia (Neeb et al. 2011). However, it is unclear whether constitutive IL-1β in brain may influence basal COX-2 expression and thus its neuromodulatory effects. Earlier studies from this group showed inhibition of COX-2 lowered the acute convulsive seizure threshold in wild-type mice and in mice lacking IL-1 signaling similarly, indicating the possibility of COX-2 functioning downstream of IL-1 signaling (Claycomb, Hewett, and Hewett 2012). These evidences led us to hypothesize that constitutive IL-1β might regulate the innate seizure threshold via COX-2.

Expression of IL-1β receptor, IL-1R1 is highly concentrated in the hippocampus (French et al. 1999) and P2X7R, the purinergic receptor which may be required for physiological IL-1β release is also expressed in the hippocampus (Metzger et al. 2017). Constitutive IL-1β accumulates in CA3/CA1 pyramidal neurons and in certain populations of hippocampal neurons in culture (studied in chapter 3). Within CNS, COX-2 is constitutively expressed in certain glutaminergic neuronal population including pyramidal cells of CA3 and is induced in granule cells of DG post seizure (Claycomb, Hewett, and Hewett 2011; Gong and Hewett 2018). Therefore, our study will focus on hippocampus as the site of action for neuromodulatory IL-1β expression and function.

4.3 Materials and Methods

4.3.1 Mice

CD-1 mice: Male 6-7-week-old CD-1 mice for *in vivo* studies were obtained from Charles River Labs (Wilmington, MA) and housed three per cage for at least one week prior to use. For studies using hippocampal neuron cultures, pregnant female CD-1 mice were purchased from Charles River Labs and housed singly upon arrival at Syracuse University.

IL-1R1 mutant mice: Mutant C57BL/6J mice lacking a functional gene for IL-1R1 (Il1r1) were obtained from The Jackson Laboratory [Stock #003245,(Glaccum et al. 1997)] and maintained as a breeding colony in the Syracuse University vivarium as described previously (Claycomb, Hewett, and Hewett 2012). Details of breeding and genotyping procedures are provided in section 2.3.1 of Chapter 2. Brains of the mice used for PTZ study from Chapter 2 were utilized for immunohistochemical and prostaglandin study herein.

4.3.1.1 Animal housing: as described in section 2.3.1.1 of chapter 2.

4.3.1.2 Anesthesia: Mice for all *in vivo* studies were anesthetized with 120 mg/kg ketamine, 20 mg/kg xylazine, intraperitoneally in saline (1X). P0-P1 pups for hippocampal neuronal dissection were anesthetized using isoflurane (1 ml on a gauze pad in a closed container from approximately 8 pups).

4.3.2 Pentylenetetrazol (PTZ) induced acute seizure paradigm

Detailed functioning, usage, and injection technique for the acute seizure paradigm have been described in section 2.3.3 of Chapter 2. All acute seizure study used mice injected with saline (1X) as control.

4.3.2.1 Dose-response study: Separate cohorts of CD-1 mice were treated with a single dose of either 50,55 or 60 mg/kg PTZ and seizure behavior scored using our 5 point severity scale as described previously: 0, normal behavior; 1, hypo-mobility; 2, myoclonus; 3 and 4, convulsion without and with loss of righting, respectively (Claycomb, Hewett, and Hewett 2011). Additionally, the incidence of convulsion (% of mice exhibiting convulsion, score ≥3) was also quantified for this study. The dose with spread of seizure response and maximally effective doses (>90% mice had convulsive seizure) for male CD-1 mice were 55mg/kg and 60 mg/kg respectively (Fig 6.1).

4.3.2.2 Acute seizure paradigm: To model neuronal excitation uniformly within all mice, a dose which effectively generated convulsive seizure was chosen. Based on results from the dose-response analysis performed; acute seizure activity was induced by a single dose of 60 mg/kg PTZ on CD-1 mice as it induced convulsive seizure in >90% of mice.

4.3.3 Primary hippocampal neuron cultures

4.3.3.1 Preparation

The method of culturing hippocampal neurons has been broadly discussed in chapter 5 of this dissertation thesis. Primary hippocampal neurons were cultured following the method discussed briefly in the section 3.3.2 of chapter 3.

4.3.3.2 Cell culture studies

All studies with primary hippocampal neuron cultures were performed at 14-15 days *in vitro* (DIV). For studies with bicuculline methobromide, a 100mM stock solution was prepared in DMSO and frozen at -20ºC. This was diluted in growth medium and administered to cells at a final concentration of 100µM.

For IL-1R1 neutralization, 1µg/µl neutralizing IL-1R1 Ab (details in table 1.3) (stock conc. 1mg/ml) was diluted in growth medium and administered to the wells designated for neutralization treatments. To study effect of neuronal excitation in absence of/lowered IL-1 signaling, bicuculline (100µM) was administered following 2 hours neutralizing antibody treatment for two hours. Wells were replaced with fresh medium prior to the studies.

4.3.4 Quantitative PCR analysis

4.3.4.1 Sample collection (*in vivo*): Mice with seizure score of 3 or 4 and their saline controls were included in this study. Fully anesthetized mice were exsanguinated and perfused transcardially with cold phosphate-buffered saline (PBS) and the hippocampi from both hemispheres were removed and stored in -80°C immediately. Before RNA extraction, one hippocampal side (studies primarily was done with left hemisphere, right was kept on reserve) for each mouse sample was homogenized in 250µl PBS on ice, aliquoted into two tubes and each dissolved in 1mL TriZol Reagent in labelled centrifuge tubes and immediately stored at -80°C prior to RNA extraction.

4.3.4.2 RNA extraction and cDNA synthesis: As described in the sections 3.3.7.2 and 3.3.7.3 of chapter 3.

4.3.4.3 Quantitative PCR: Total RNA was purified and reverse transcribed as described previously (Hewett et al. 1999) and expression of c-Fos, IL-1β, IL1R1,IL1RacP, Caspase-1 and P2X7R was quantified with a real-time PCR detection system (Mastercycler ep RealPlex^2 Real-Time PCR System, Eppendorf)) using Applied Biosystems TaqMan Universal PCR Master Mix and FAM-MGB dye gene expression assay probes. Relative expression was quantified using the comparative cycle threshold method, ΔΔCt (Livak and Schmittgen 2001), where individual Ct values were normalized to the β-actin Ct value from the same sample then with a control sample

Ct value (calibrator) to determine the relative fold changes in mRNA. To assess primer efficiency for each analysis, qPCR reactions were performed on a dilution series of known cDNA concentrations (12.5, 25, 50, 100, and 200 ng/reaction, cDNA generated from saline-treated CD-1 mice hippocampus). Efficiency across all input concentrations was confirmed (slope of relative efficiency plot <0.1) between all primer pairs and housekeeping gene (β-actin) primer pair. The expression assay probes (Thermo Fisher Scientific) were cFos, IL-1β, IL1r1, IL1RaP, Caspase-1, P2X7R, COX-2 and β-actin (details in table 1.5).

4.3.7 Immunofluorescence analysis of protein

4.3.7.1 Immunohistochemistry: As described in the section 3.3.4 of chapter 3.

4.3.7.2 Immunocytochemistry: As described in the section 3.3.5 of chapter 3.

4.3.7.3 Antibodies for IHC and ICC.

Primary: **IL-1β, IL-1R1, NeuN, MAP-2, COX-2.**

Secondary: Donkey anti Rabbit Alexa Fluor 488, Donkey anti-Mouse Alexa Fluor 594, Goat anti Hamster DyLight 405. Details of antibodies and their usage are provided in table 1.3.

4.3.7.4 Quantification of immunoreactivity.

All brain section images of subregions of hippocampi were converted to 8-bit gray scale and fluorescent intensity was quantified using NIH FIJI (Image J2). For COX-2 immunoreactivity, the maximum and minimum thresholds were set to 26 and 255, using the auto thresholding technique. Results from two images from each brain section were averaged and the mean fluorescent integrated density was calculated in the hippocampal sub-regions using methods described previously (Gong and Hewett 2018).

To quantify fluorescence intensity in hippocampal neurons in culture, maximal and minimal threshold was set similarly for individual study using auto thresholding technique and the

total fluorescent integrated density for each field was calculated. Alongside total number of neurons in each field were counted using cell counter plugin in FIJI (3 fields/well, 3 wells/plate X 2 replicates). Total fluorescence integrated density was divided by the number of neurons for individual fields which was then quantitatively represented as fluorescence intensity per neuron. For visual representation, images from individual studies were processed identically using Adobe Photoshop.

4.3.8 IL-1β Immunoblot analysis: As described in section 3.3.8 of chapter 3.

4.3.9 Enzyme linked Immunosorbent Assay (ELISA)

4.3.9.1 IL-1β ELISA

4.3.9.1.1 *In vivo* sample collection: Mice with seizure score of 3 or 4 and their saline controls were included in this study. Prior to brain dissection, all mice were fully anesthetized with 100/10mg/kg ketamine/xylazine, respectively, i.p. For time-course analyses of IL-1β protein expression, CD-1 mice, were exsanguinated 15, 30, 60, and 120 minutes following PTZ injection by transcardial perfusion with ice-cold PBS and the hippocampi were removed. Hippocampal tissue was homogenized in Cell Lysis Buffer 2 (Part#895347, R & D Systems) and the supernatant was collected after centrifuging at 13000*g for 30 minutes and stored at -80°C prior to using.

4.3.9.1.2 ELISA: A commercially available ELIZA kit was used to quantify IL-1β in hippocampal tissue of mice brain (Quantikine mouse IL-1β ELISA). Total protein concentration was quantified using commercially available kit (BCA) and supernatant were adjusted to 1µg/µL protein with lysis buffer. IL-1β concentrations were determined from a standard curve (1.25pg/ml- 80pg/ml) by diluting the known concentration provided by the manufacturer.

4.3.9.2 Prostaglandin (PGE_2) assay

4.3.9.2.1 Impeding endogenous COX-2 production due to surgery: Saline or PTZ treated mice were immediately anesthetized after injection or after PTZ induced convulsion, respectively. Peritoneal cavity of anesthetized mouse was opened via surgery and injected with indomethacin (10mg/kg) diluted in PBS into the inferior vena cava. After 3 minutes, transcardial perfusion was conducted with 12-15 ml ice-cold 1X PBS followed by 10 ml indomethacin (20mM diluted in 1X PBS). The hippocampi were quickly removed and snap-frozen in liquid nitrogen.

4.3.9.2.2 Homogenization of hippocampal tissue: Hippocampal tissue was homogenized in 100-150µl homogenization buffer (containing 10% v/v 10mM EDTA, 0.1% v/v 10mM Indomethacin and 1mM KH2PO4 in ultrapure water) and supernatant was collected after centrifuging at 12000*g for 20 minutes and stored at -80^{o}C prior to use.

4.3.9.2.3 PGE_2 ELISA: Total protein concentration was quantified using commercially available kit (BCA) and supernatants were adjusted to 0.25µg/µL protein with homogenization buffer. PGE_2 concentrations were determined using workbook provided by manufacturer from a standard curve (31.25pg/ml- 4000pg/ml) by diluting the known concentration provided by the manufacturer.

4.3.10 Statistical analysis

Data were analyzed using GraphPad Prism, Version 8.4.1 (GraphPad Software, Inc., La Jolla, CA, U.S.A.). Statistical tests are described in figure legends. Statistical significance was set to $p<0.05$. Statistical tests described in figure legends. Behavioral data associated with scoring of seizure are reported as median seizure score and incidence of a convulsive seizure. Latency to convulsion after acute PTZ injection between genotypes was assessed using a two-tailed Mann–Whitney test. Datasets representing proportions (incidence of convulsions) were analyzed using a

two-tailed Fisher's exact test. For q-PCR, statistics were performed on the logarithmic transformation of $2^{-\Delta\Delta CT}$ values, and analyzed with Kruskal Wallis test followed by uncorrected Dunn's test. For t-test, one-way ANOVA, two-way ANOVA, in addition to *p* values, F values were determined for equal variance. (for ANOVA, F (DFn, DFd) where DFn is the numerator of df (degree of freedom) and DFd is the denominator of df and for unpaired t-test, t values and df, in addition to *p* values). In case this *p*-value was significant, the Welch t-test was done instead of unpaired t-test and Brown Forsythe test was done instead of one-way ANOVA to account for this variance. For *in vivo* studies comparing immunofluorescence , two-way ANOVA were followed by Bonferroni's multiple comparison test. For comparing PG levels in mice hippocampus and for *in vitro* studies, one-way or two-way ANOVA were followed by Fisher's uncorrected LSD test.

4.4 Results

4.4.1 PTZ induced acute seizure: Dose-response study

PTZ elicits seizure and convulsion in a dose-dependent manner (Claycomb, Hewett, and Hewett 2011). The threshold and maximally effective doses for my studies was assessed in a dose-response analysis of CD-1 mice by treating the mice with either 50 mg/kg (N=14), 55 mg/kg (N=6) or 60 mg/kg (N=16) b.w. of PTZ and seizure behavior was scored using our 5-point severity scale as described in methods. The threshold (dose with intermediate population response) (33%) and maximally effective doses [PTZ dose inducing convulsive seizure in >90% mice) determined for male CD-1 mice were 55mg/kg and 60 mg/kg respectively (Fig. 4.1). 60mg/kg PTZ was chosen for the following seizure studies in mice because it induced convulsions in with an incidence of >90%.

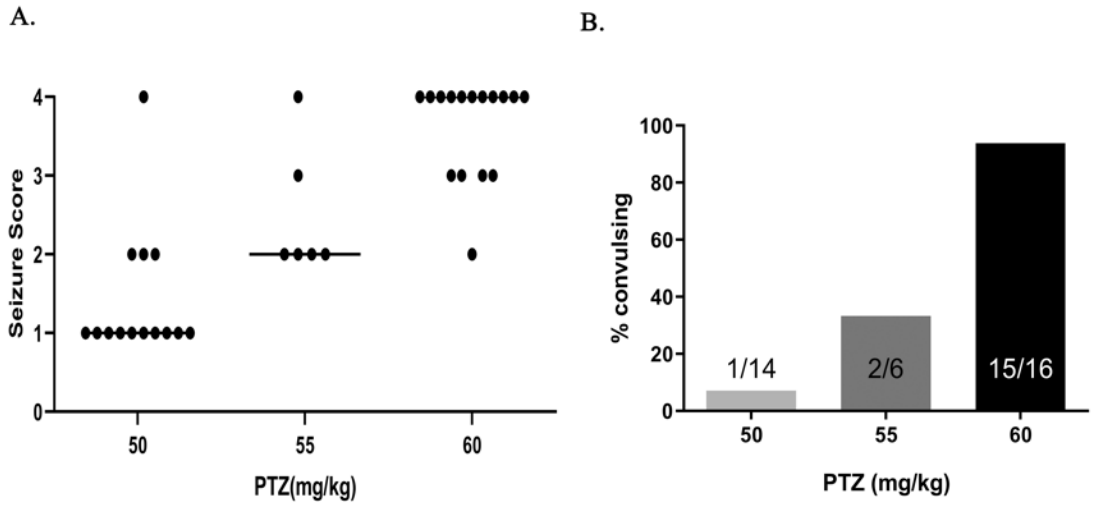


Fig.4.1. Identification of PTZ dose for acute convulsive seizure threshold analyses.

Dose response analysis of CD-1 mice were conducted by treating mice with either 50mg/kg (N=14), 55mg/kg (N=6) or 60mg/kg (N=16) b.w.

A. Seizure severity. Each point represents the maximum seizure score for an individual mouse, horizontal line represents median seizure score for that dose.
B. Incidence of convulsions. The number of mice exhibiting a convulsive seizure in A is expressed as a % of total mice injected with PTZ for each treatment group (ratio in the bar is the raw data).

4.4.2 Effect on IL-1β ligand and signaling components in mice hippocampus with PTZ induced seizure activity

To test whether intensive neuronal activity in mice induced or altered IL-1β mRNA and protein level, CD-1 mice were challenged with 60mg/kg PTZ and their seizure response were assessed. IL-1β mRNA levels at different time points post injection in hippocampal tissue was compared to saline treated mice tissue (15-, 30-, 60- and 120-min post PTZ injection). IL-1β mRNA level elevated significantly in hippocampus immediately after convulsion (4.5 times compared to saline treated hippocampus 15 min post injection). IL-1β mRNA restored to basal level in hippocampus by 60 mins following injection (Fig.4.2). This transient increase in IL-1β mRNA level was consistent with elevated c-Fos mRNA level, used as surrogate marker of excitatory neuronal activity (Morgan et al. 1987). However, no immediate significant changes were identified in the mRNA levels of IL-1β receptor, IL-1R1 and its accessory protein, Il-1RacP (Figure 4.3 A and B) and in P2X7R (the purinergic receptor required for IL-1β release) mRNA level (Figure 4.3 C). However, Caspase-1 mRNA, the proteolytic enzyme required for cleavage of IL-1β to its functional form and for its secretion, elevated 4 times by 60 mins and remained elevated at 120 mins (Figure 4.3 D).

To test if the increase in IL-1β mRNA translated to change in protein level, IL-1β level was measured using ELISA in hippocampal tissue of mice having PTZ induced convulsion in a time course study (15-, 30- and 60-min post injection). ELISA analysis demonstrated constitutive IL-1β protein in low pico-gram level indicating physiological presence of IL-1β in hippocampal tissue. Time course study showed no immediate alteration in IL-1β protein levels with intensive neuronal activity; however, it did not affect the basal levels (Fig 4.4).

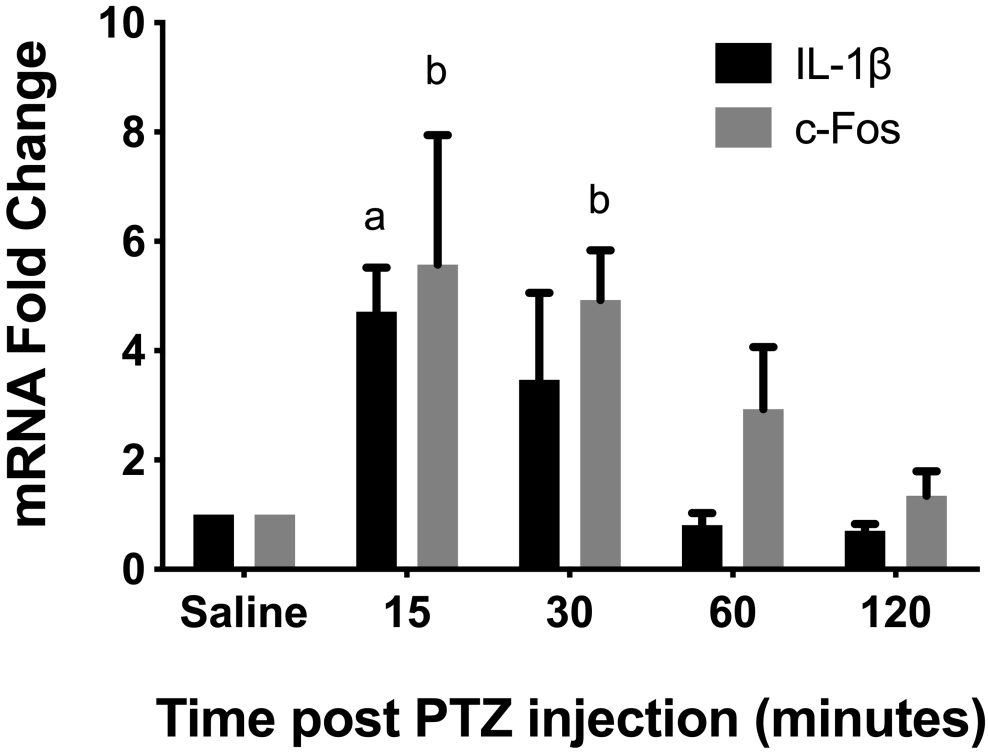


Fig. 4.2. IL-1β mRNA level in hippocampus changes in parallel to basal excitatory neuronal activity.

Time course (15 min (N=6), 30 min(N=6), 60 min(N=5) and 120 min (N=4)) of IL-1β and c-Fos mRNA expression following PTZ induced convulsive seizures in mice (60mg/kg b.w.) compared to saline treated control mice (N=6) . A 2-way ANOVA followed by uncorrected Fisher's LSD comparisons test was performed on log transformed data showed significant difference in mRNA levels with treatment from respective vehicle-treated controls (*, $p = 0.0021$).

(For IL-1β mRNA, *, $p = 0.0041$, Kruskal-Wallis test with multiple comparison by uncorrected Dunn's test. Significance denoted by "a" ,*, $p= 0.0141$, saline vs. 15 min, $p = 0.8319$, saline vs. 30 min, $p= 0.4663$, saline vs. 60 min and $p=0.2547$, saline vs. 120 min.
For c-Fos mRNA, *, $p = 0.0198$, Kruskal-Wallis test with multiple comparison by uncorrected Dunn's test. Significance denoted by "b", *, $p= 0.0144$, saline vs. 15 min, *, $p = 0.0068$, saline vs. 30 min, $p= 0.1674$, saline vs. 60 min and $p=0.8252$ and saline vs. 120 min.)

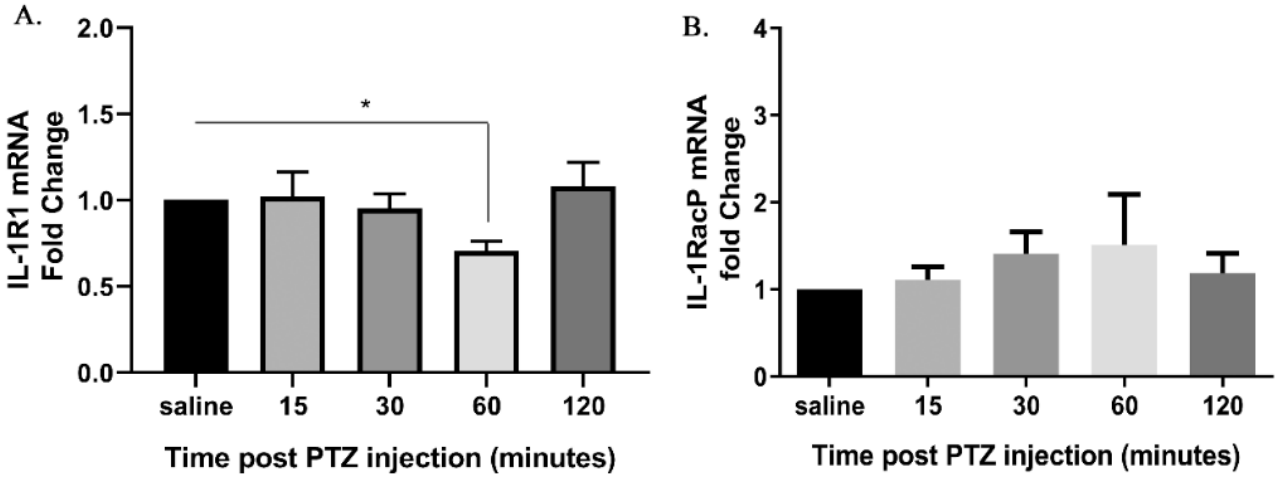
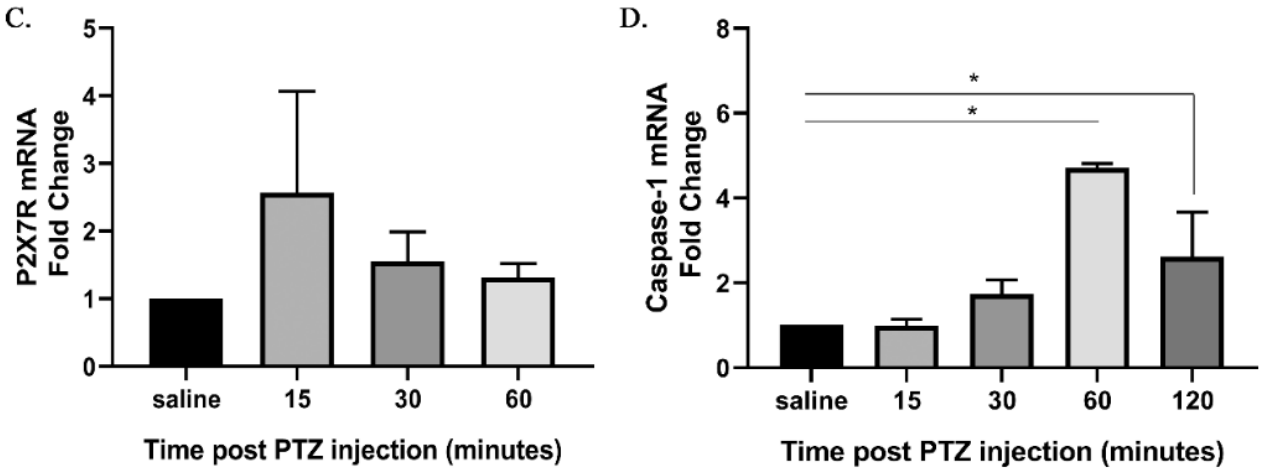


Fig.4.3. Effect on IL-1β signaling components' mRNA in mice hippocampus with acute convulsive seizure.

A. Time course (15 min (N=7),30 min (N=7), 60 min (N=4) and 120 min (N=5)) of IL-1R1 mRNA expression following PTZ induced convulsive seizures in mice (60mg/kg b.w.) compared to saline-treated control mice (N=6).
For IL-1R1 mRNA, p = 0.1444, Kruskal-Wallis test with multiple comparison by uncorrected Dunn's test, p= 0.6278, saline vs. 15 min, p = 0.3323, saline vs. 30 min, *, p= 0.0186, saline vs. 60 min and p=0.9379 saline vs. 120 min.

B. Time course (15 min (N=6),30 min (N=5), 60 min (N=4) and 120 min (N=5)) of IL-1RaP mRNA expression following PTZ induced convulsive seizures in mice (60mg/kg b.w.) compared to saline-treated control mice (N=5).
For IL-1RaP mRNA, p =0.833, Kruskal-Wallis test with multiple comparison by uncorrected Dunn's test, p= 0.548 saline vs. 15 min, p = 0.2441, saline vs. 30 min, p= 0.4159, saline vs. 60 min and p=0.49, saline vs. 120 min.



C. Time course (15 min (N=3),30 min (N=3), 60 min (N=3)) of P2X7R mRNA expression following PTZ induced convulsive seizures in mice (60mg/kg b.w.) compared to saline-treated control mice (N=3).
For P2X7R mRNA, $p = 0.6255$, Kruskal-Wallis test with multiple comparisons by uncorrected Dunn's test, $p= 0.2097$, saline vs. 15 min, $p = 0.3048$, saline vs. 30 min and $p=0.2542$ saline vs. 60 min.

D. Time course (15 min (N=4),30 min (N=4), 60 min (N=3)and 120 min (N=4)) of Caspase-1 mRNA expression following PTZ induced convulsive seizures in mice (60mg/kg b.w.) compared to saline treated control mice (N=4).
For Caspase-1 mRNA, *, $p = 0.0135$, Kruskal-Wallis test with multiple comparison by uncorrected Dunn's test, $p= 0.8007$, saline vs. 15 min, $p= 0.0583$, saline vs. 30 min and *, $p=0.0035$, saline vs. 60 min and *, $p=0.0373$, saline vs. 120min.

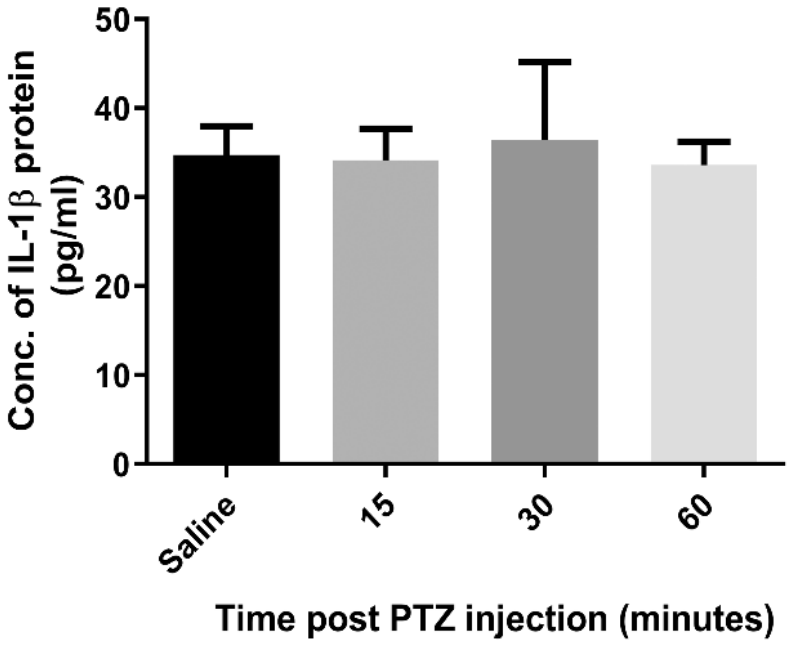


Fig.4.4. IL-1β protein expression is not altered by acute convulsive seizure.

IL-1β level was quantified by ELISA in hippocampal homogenates of mice either treated with saline (N=5) or with PTZ injection [15min (N=5), 30 min (N=4), and 60 min (N=5)]. IL-1β level between time points were compared by Brown-Forsythe ANOVA test [#] (p=0.9815, F*(DFn, DFd)=0.05496 (3, 6.025) followed by unpaired t with Welch correction (p= 0.9068, saline vs. 15 min, p = 0.8648, saline vs. 30 min and p=0.07965, saline vs. 60 min).

[#] Brown-Forsythe ANOVA test was done instead of one-way ANOVA as significant difference was found in variance Brown-Forsythe test (**, p= 0.0096, F(DFn, DFn)=5.484 (3,15)).

4.4.3 Effect on IL-1β ligand in cultured hippocampal neurons with bicuculline induced neuronal excitation

To assess this *in vitro*, cultured primary hippocampal neurons were treated with bicuculline to shift E/I balance to favor excitation by disinhibition. Previous study in chapter 3 have shown constitutive IL-1β immunoreactivity in subset of cultured neurons where IL-1β is expressed in cell body as perinuclear puncta and in neuronal processes. Similar IL-1β immunoreactivity

pattern was observed in vehicle treated cells and this level remained same with 4 hours bicuculline treatment (Fig.4.5).

Immunoblot analysis of hippocampal cell lysates showed corresponding results. Constitutive presence of only 31kD pro-IL-1β protein in cultured hippocampal neurons was seen whose level did not alter with bicuculline induced neuronal activity. Alongside, there was no evidence of 17kD processed IL-1β peptide in both vehicle and bicuculline treated cells (Fig.4.6).

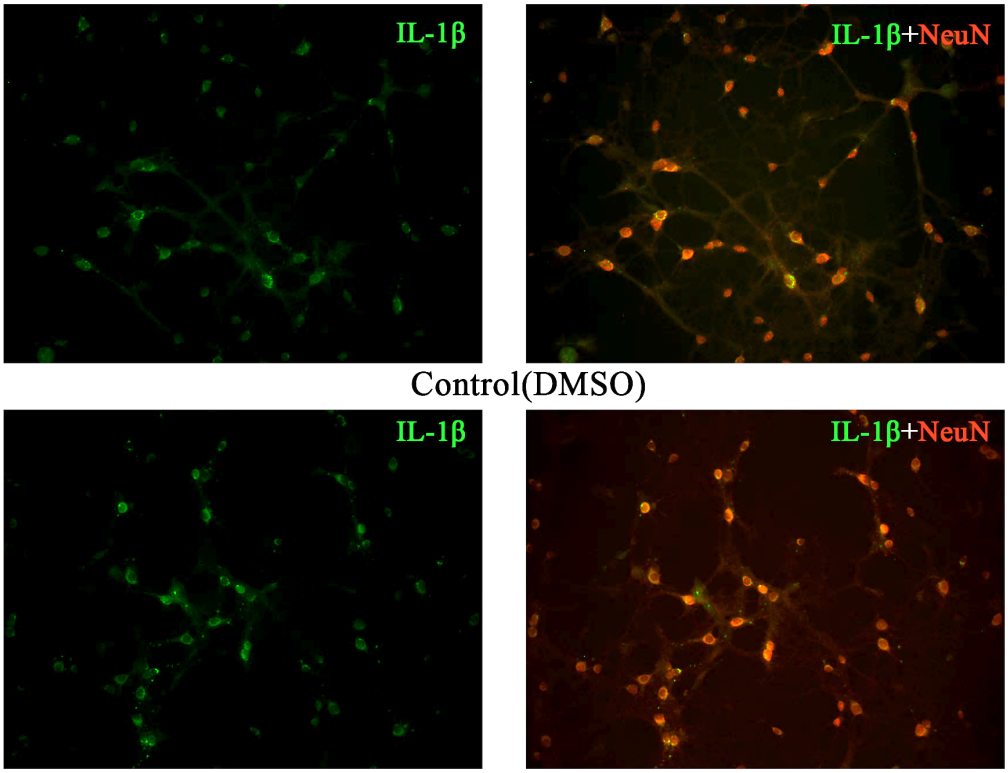


Fig.4.5A

(continued)

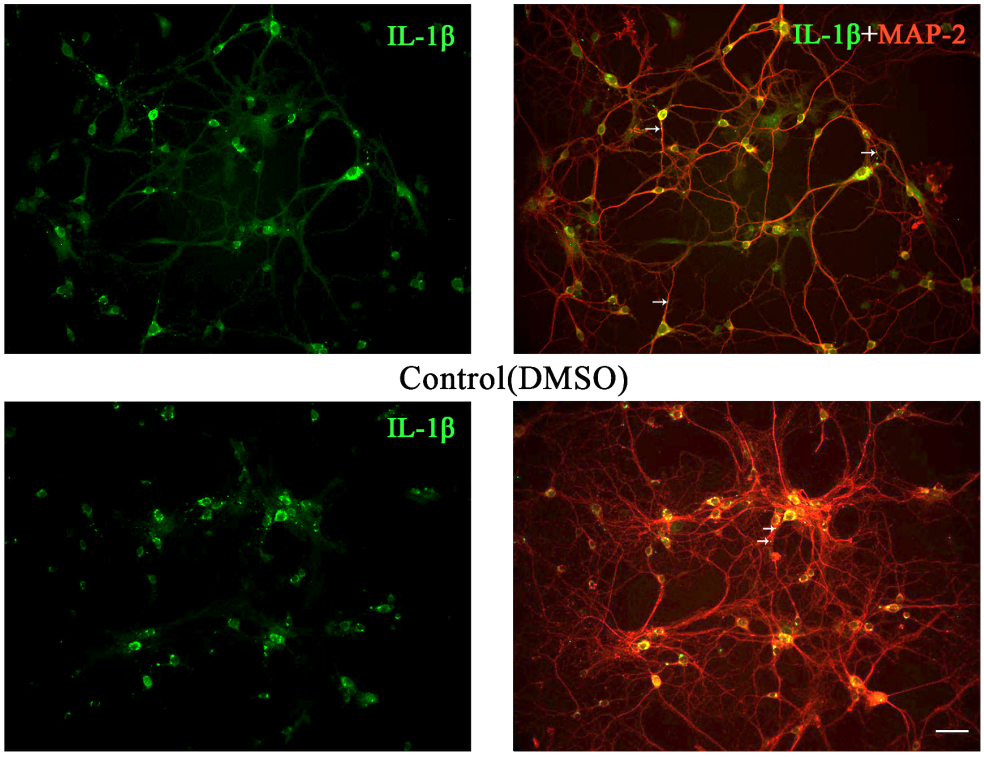


Fig.4.5B

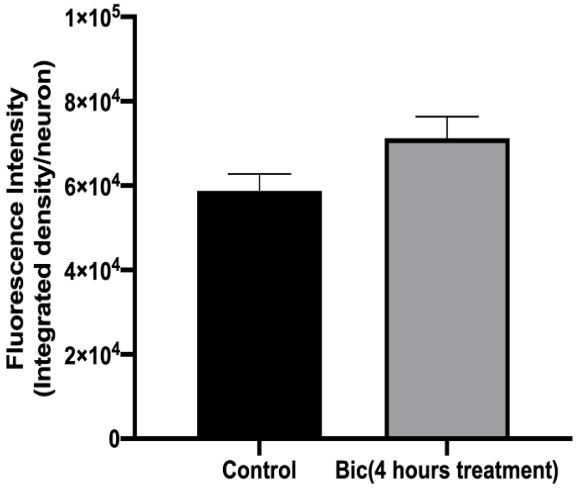


Fig.4.5C

Fig.4.5. IL-1β protein expression is not altered by neuronal excitation in cell bodies and neuronal processes of hippocampal neurons.

Representative photomicrographs of cultured hippocampal neurons in culture (DIV 14) demonstrating IL-1β (green) immunoreactivity co-stained with **A.** neuronal marker NeuN (red) and **B.** neuronal processes MAP-2 (green) in control and bicuculline treatments. (20X objective). Scale bar = 25µm. White arrows show IL-1β immunoreactivity in the neuronal processes.

C. IL-1β fluorescence intensity per neurons was quantified in hippocampal neuronal cells as described in materials and methods for control and bicuculline treatment after 4 hours treatment and analyzed using unpaired t test (p=0.0626, t=1.992, df=36).

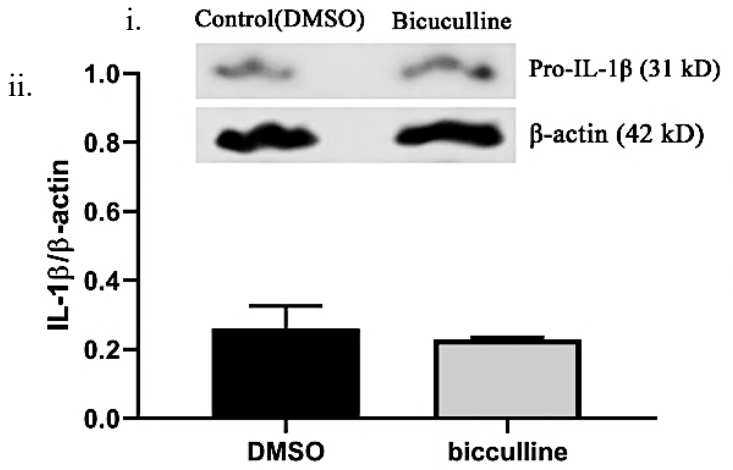


Fig.4.6: IL-1β protein expression is not altered by neuronal excitation *in vitro*.

Immunoblot analysis using anti-IL-1β and anti-β-actin antibodies was performed on cell lysates harvested after 4 hours following treatment with vehicle (DMSO) or Bicuculline as described in Materials and Methods.
i. Representative blot. **ii.** Fluorescence intensity of IL-1β in cell lysate treated either with bicuculline (N=3) or its vehicle (DMSO) (N=3) for 4 hours was quantified, normalized to β-actin fluorescence intensity and analyzed by unpaired t test (p=0.9815, t=2.277, df=6). Equal variance was validated by the F test.

4.4.4 Effect of IL-1R1 gene deletion on basal and induced COX-2 expression in mouse hippocampus

My studies have shown, P2X7R antagonism (possibly through blocking of IL-1β release) elevates basal COX-2 mRNA in hippocampal neurons (Fig. 3.8, Chapter 3). In mouse with genetic deletion of IL-1 signaling, it was reasonable to test if this altered signaling may affect COX-2 mRNA level. No genotype dependent changes in COX-2 mRNA level in hippocampus was observed between genotypes (Fig.4.7).

As suggested in the introduction, neuronal COX-2 expression and/or activity has been shown to be regulated via exogenous IL-1β treatment indicating COX-2 may function to downstream of IL-1β signaling. However, it is unknown if constitutive IL-1β affects basal COX-2 expression and/or activity. To test this hypothesis, COX-2 immunoreactivity was observed in saline treated

wild type and IL1r1 mutant littermate mice. Constitutive neuronal COX-2 expression is observed in glutaminergic neurons of cortex and in subsets of CA3 pyramidal neurons of mice (Claycomb, Hewett, and Hewett 2011; Gong and Hewett 2018). The current study was consistent with this result in the wild type littermates. Furthermore, constitutive COX-2 immunoreactivity was 36% lower in CA3 subregion of KO mice compared to the WT littermates (Fig.4.8), indicating absence of functional IL-1 signaling affected basal COX-2 expression in mice hippocampus.

To test the effect of IL-1r1 gene deletion on neuronal activity induced COX-2 expression, wild type and IL1r1 mutant littermate mice were given 43.5mg/kg PTZ and its seizure behavior was observed (studies in section 2.4.2 of Chapter 2). To understand effect of IL1r1 deletion on induced COX-2 expression, only wild type and mutant mice having convulsive seizure (seizure score of 3 or 4) were considered which kept the effect of PTZ stimulus similar across genotype. Immunohistochemical analysis of WT mice brain showed immediate elevated expression (by 1 hour) in CA3 sub-region of hippocampus and induced expression in DG and CA1 sub-regions (Fig.4.9). KO mice, on other hand, had significantly lower induced COX-2 expression in CA3 (~70% lower) and DG (~65% lower) sub-regions of the hippocampus compared to their WT littermates and no observable expression in the CA1 region (Fig.4.9 i and fluorescence intensity quantification in Fig. 4.9 ii).

Similarly, elevated COX-2 level was seen in CA3 compared to basal state and remained induced in DG sub-region of the hippocampus of WT mice following 3 hours of the convulsive seizure which is consistent with previous findings (Claycomb, Hewett, and Hewett 2011; Gong and Hewett 2018), however, activity dependent COX-2 level in IL-1r1 KO mice was significantly lower in these sub-regions compared to the WT littermates (~ 76% lower in CA3 and ~ 75%

lower in DG subregion of KO mice) (Fig.4.10 i and fluorescence intensity quantification in Fig. 4.10 ii).

Therefore, these results conclude both basal and induced COX-2 expression in absence of functional IL-1β signaling was lower.

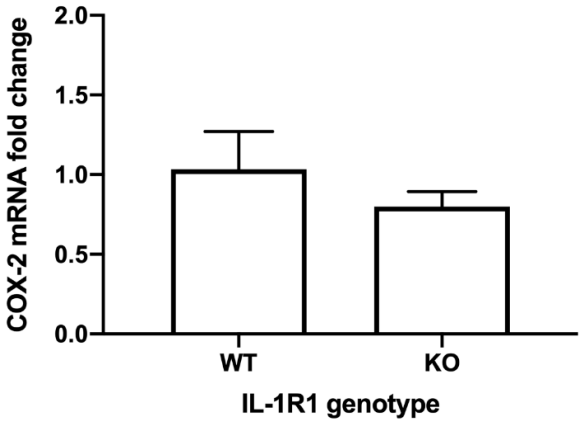


Fig.4.7. Basal COX-2 mRNA expression in the hippocampus of mice brain in mice.

Relative normalized COX-2 mRNA expression in WT and KO mice (N=3,each genotype) was compared with two-tailed, unpaired t-test (*p*= 0.4127, t=0.9135, df= 4). Fold change from one WT sample was used as calibrator and set to 1.Equal variance was validated by F test (F=6.303, DFn, DFd= 2,2).

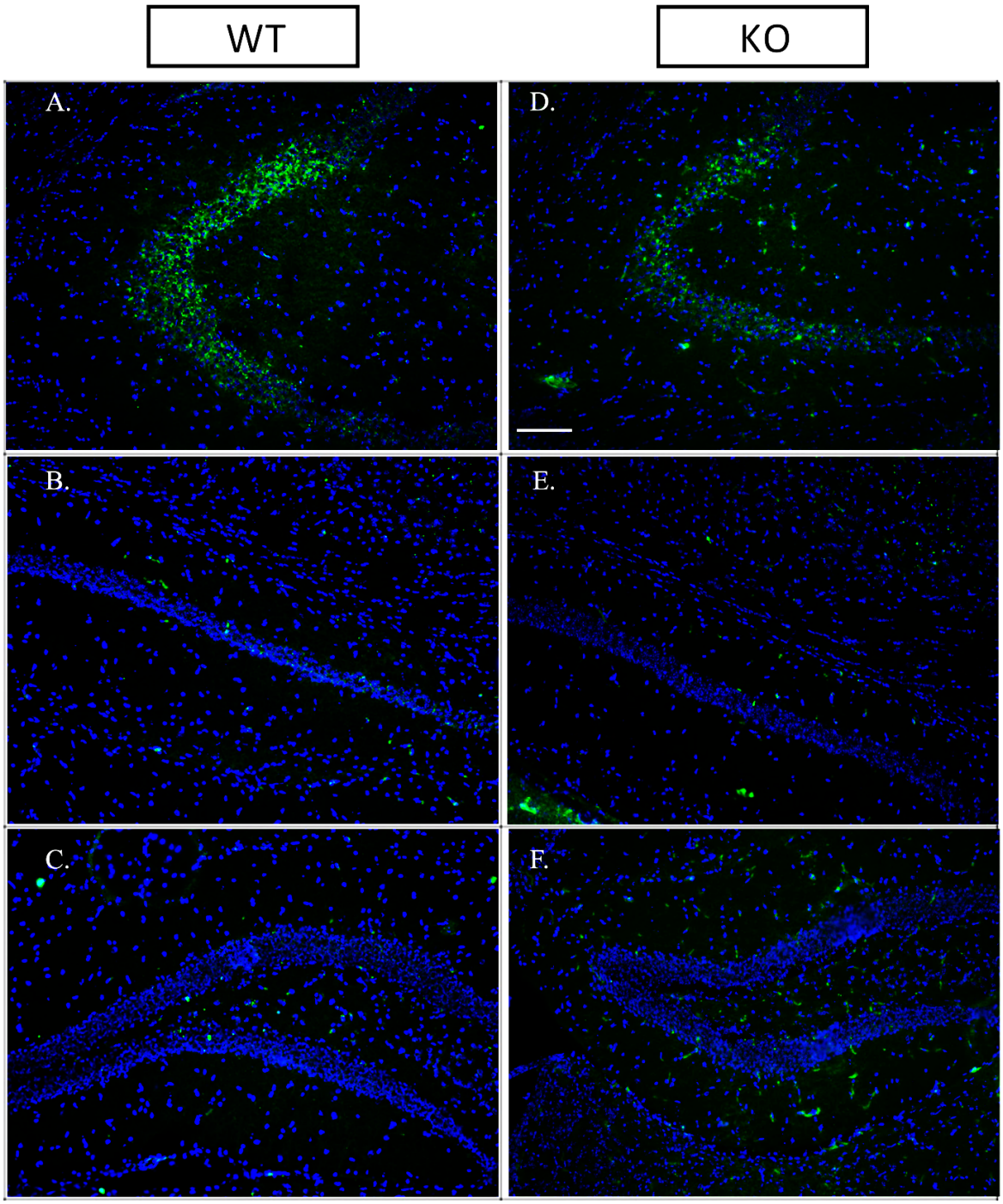
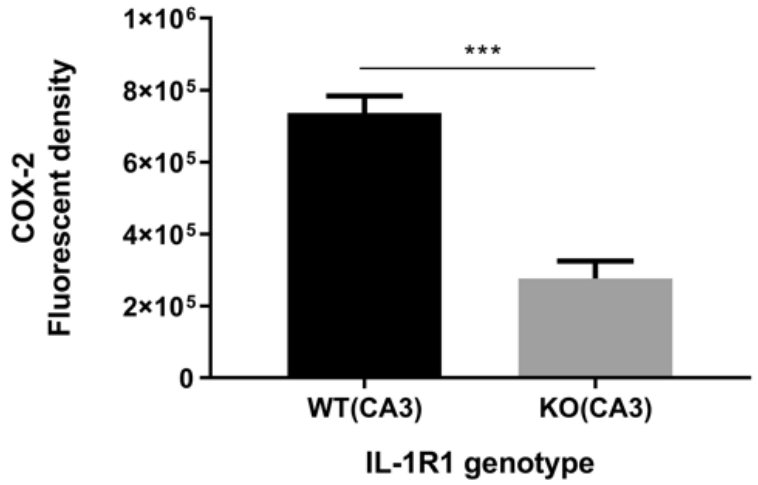


Fig.4.8. IL-1R1 gene deletion alters basal neuronal COX-2 expression in mouse hippocampus.

i. WT or KO (N= 5, each) brain sections were stained for COX-2 immunoreactivity (green), counterstained with DAPI (blue) and photomicrographs were acquired from coronal section (10X objective). Representative photomicrograph shows COX-2 immunoreactivity in (A & D) CA3, (B & E) CA1 and (C & F) DG subregion of hippocampus. Headings indicate IL-1r1 genotype for each column of images. Scale bar =100 μm.



ii. COX-2 fluorescent intensity was quantified and compared by a two-tailed unpaired t-test between the genotype for CA3 sub-regions of the hippocampus. (***, p=0.001, t=6.764, df=8 for CA3 subregion). Equal variance was validated by F test (F, DFn, DFd= 1.036, 4, 4). No COX-2 expression was detected in CA1 and DG sub-region of the hippocampus in both the genotype.

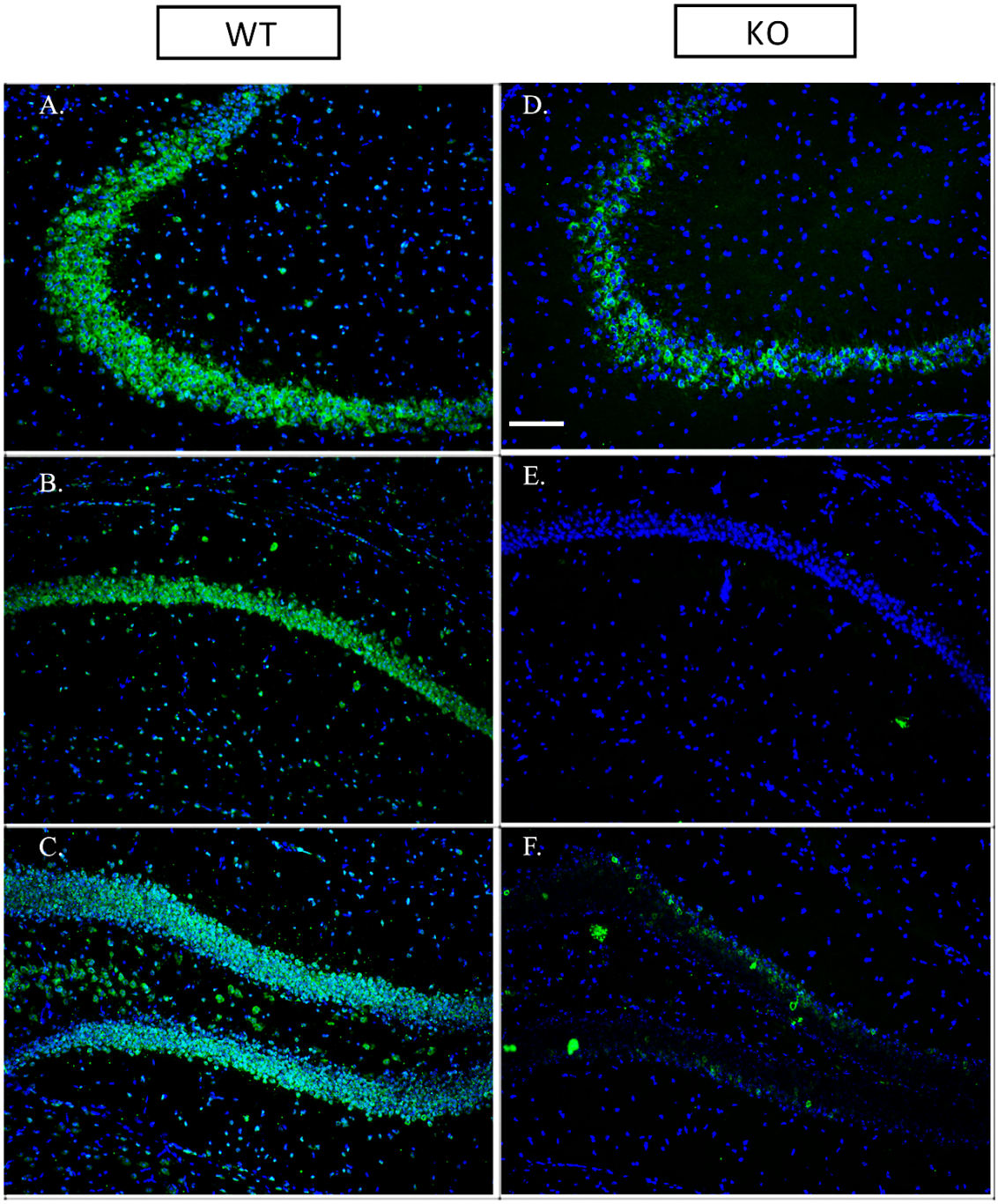
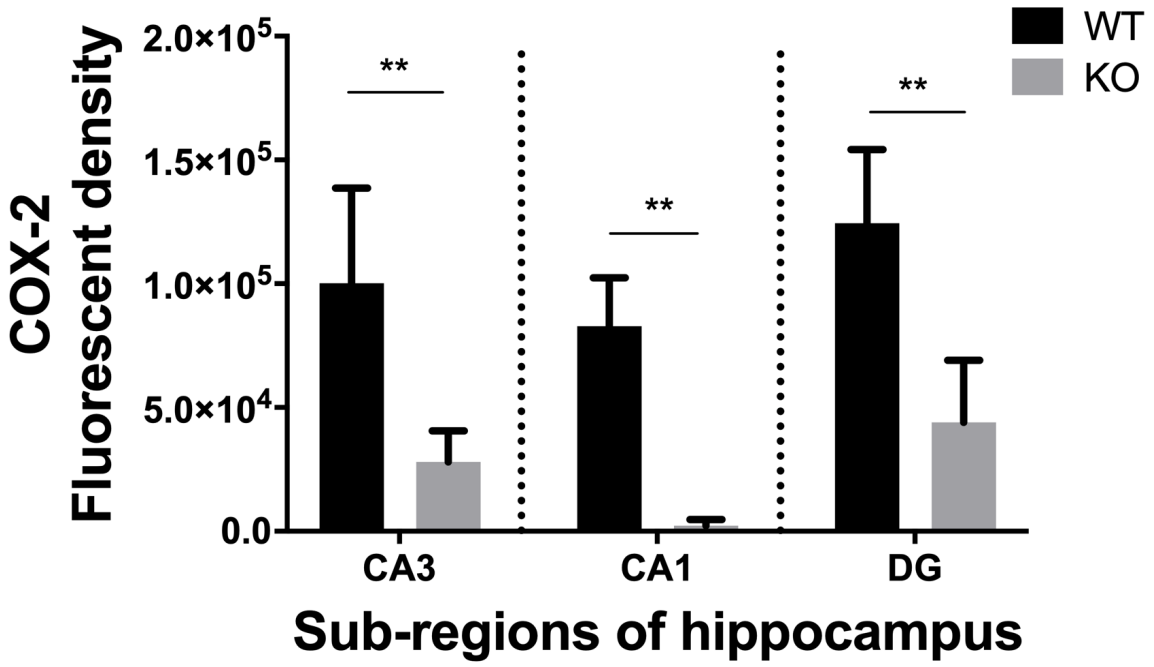


Fig.4.9. IL-1R1 gene deletion alters activity dependent neuronal COX-2 protein expression in mouse hippocampus.

One hour following PTZ injection, WT or KO (N= 5,each) mice brain sections were stained for COX-2 immunoreactivity (green) and DAPI (blue) and photomicrographs were acquired from coronal section (10X objective). Representative photomicrograph shows COX-2 immunoreactivity in (A, D) CA3, (B, E) CA1 and (C, F) DG subregion of hippocampus. Headings indicate IL-1r1 genotype for each column of images. Scale bar = 100 μm. (continued)



ii. COX-2 fluorescent intensity was quantified for all three sub-regions of hippocampus and were analyzed using 2-way ANOVA followed by Bonferroni's multiple comparisons test. Significant differences (a, between genotypes (***,p<0.001) and b, from the corresponding value in the subregions (***, p <0.001)) were observed. No COX-2 expression was detected in CA1 sub-region of hippocampus in KO mice brain. Equal variance was validated by F test.

Bonferroni's multiple comparisons tests	Significant?	Summary	Adjusted *p*-Value
WT - KO			
CA3	Yes	**	<0.0001
CA1	Yes	**	0.0005
DG	Yes	**	<0.0001

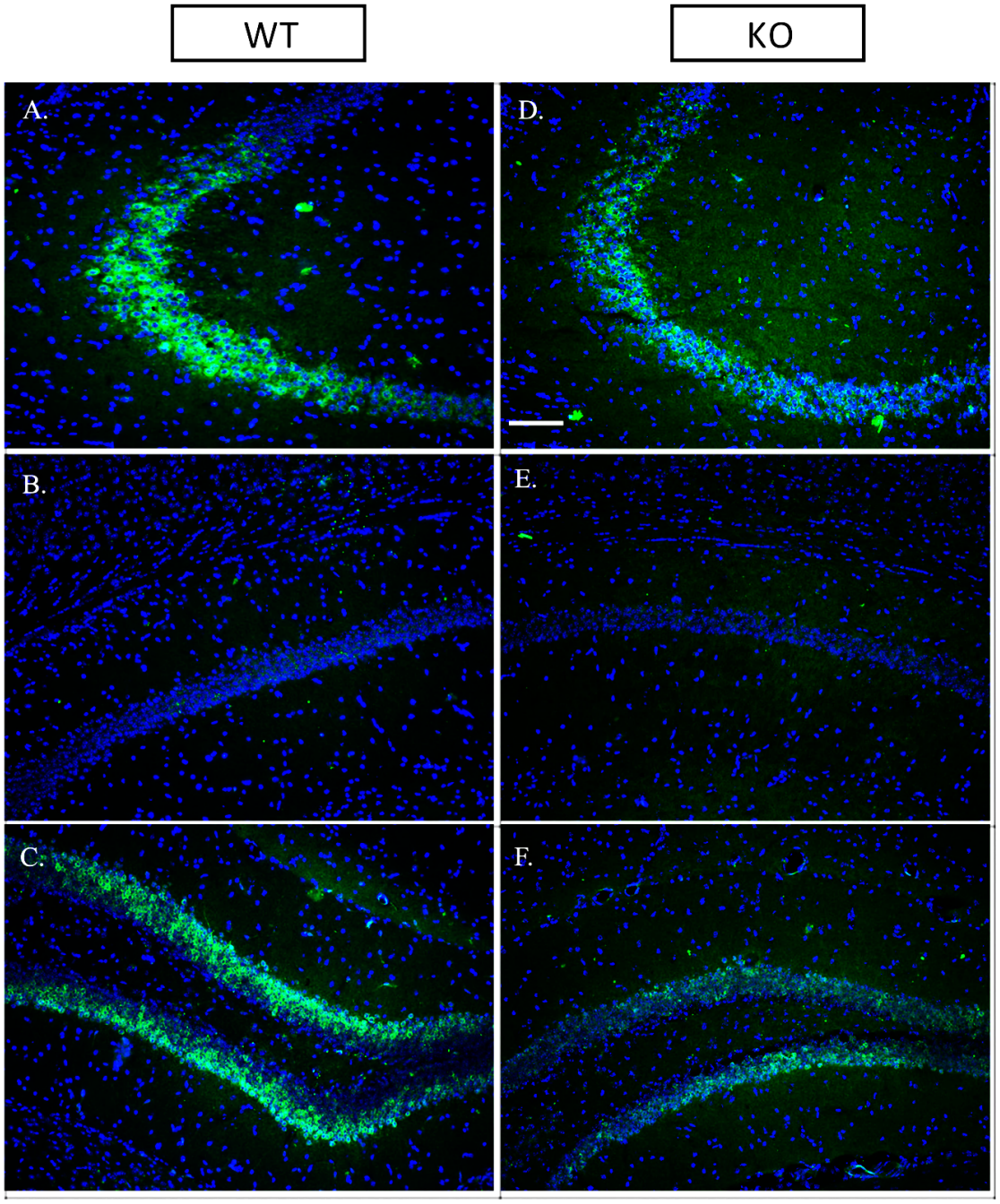
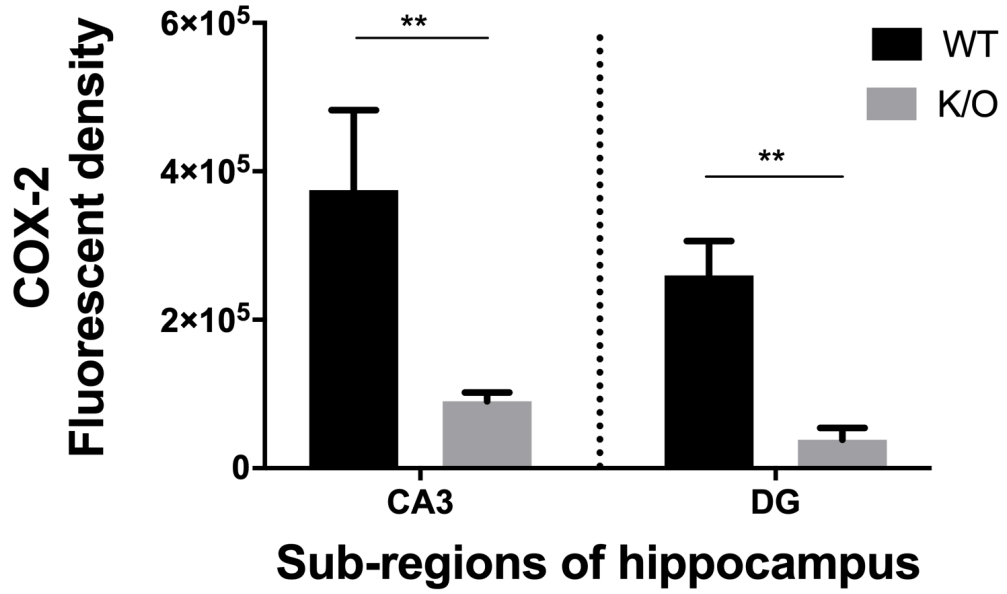


Fig.4.10. IL-1R1 gene deletion alters activity dependent neuronal COX-2 protein expression in mouse hippocampus.

Three hours following PTZ injection, WT or KO (*N*= 3) brain section was stained forCOX-2 immunoreactivity (green) counterstained with DAPI (blue) and photomicrographs were acquired from coronal section (10X objective). Representative photomicrograph shows COX-2 immunoreactivity in (A, D) CA3, (B, E) CA1 and (C, F) DG subregion of hippocampus. Headings indicate IL-1r1 genotype for each column of images. Scale bar = 100 µm.



ii. COX-2 fluorescent intensity for was quantified for the CA3 and DG sub-regions of hippocampus and were analyzed using 2-way ANOVA followed by Bonferroni's multiple comparisons test. Significant differences (a, between genotypes (*, p=0.0409) and b, from the corresponding value in the subregions (*, p <0.001)) were observed. No COX-2 expression was detected in CA1 sub-region of hippocampus of either genotype. Equal variance was validated by F test.

Bonferroni's multiple comparisons tests	Significant?	Summary	Adjusted p-Value
WT-KO			
DG	Yes	**	0.0037
CA3	Yes	**	0.0007

4.4.5 Effect of excitatory neuronal activity on COX-2 mRNA and protein expression in hippocampal neurons.

Section 3.4.4 in chapter 3 demonstrated the relationship between COX-2 mRNA expression and neuronal excitation. Cultures of hippocampal neurons were treated with the $GABA_A$ receptor antagonist, bicuculline (Fig 3.8 D). Compared to vehicle-treated cultures, bicuculline induced a 6-fold elevation in COX-2 mRNA expression alongside 7-fold increase in cFos mRNA (surrogate marker for neuronal excitation) expression (Fig. 3.8 B, respectively).

Similarly, a different set of cultured hippocampal neurons either pretreated (for 30 mins) with or without MK-801(non-competitive NMDA antagonist) and were then treated with bicuculline ($GABA_A$ antagonist) or its vehicle for 60 min. MK-801 treatment, similar to APV treatment, did not change basal COX-2 or c-Fos level. Compared to the culture which received no or MK-801 treatment, COX-2 and c-Fos MRNA were elevated approximately 4 and 3.5 folds, respectively in neurons treated only with bicuculline. However, cultures which received bicuculline following MK-801 pre-treatment significantly attenuated COX-2 and c-Fos mRNA level, implying elevation in COX-2 mRNA level due to excitatory neuronal activity was NMDA receptor dependent (Fig. 4.11).

Certain population of hippocampal neurons in culture expressed COX-2 endogenously (Fig 4.12 A, Control). Alongside elevation in COX-2 mRNA level with excitatory neuronal activity, hippocampal neurons also showed an elevation in COX-2 protein expression following bicuculline treatment (2 hours and 4 hours, Fig. 4.12 A). Induced COX-2 immunoreactivity was significantly elevated within 2 hours of treatment which lowered gradually to basal level by 4 hours (as quantified in Fig. 4.12 B). Alongside, significant elevation in total COX-2 intensity, % of neurons expressing COX-2 was also significantly higher at 2 hours post bicuculline treatment (Fig. 4.12 C).

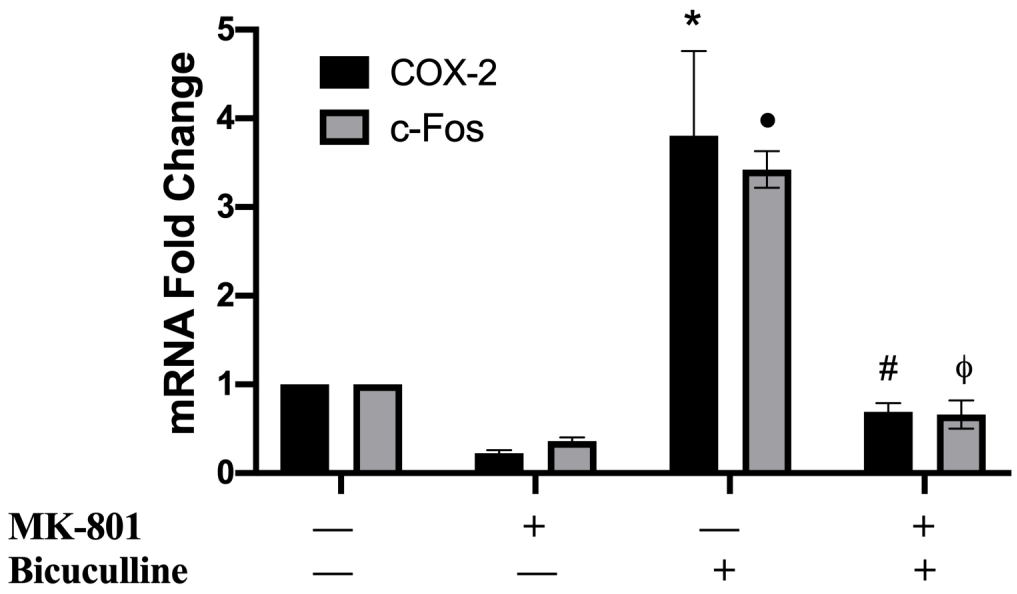
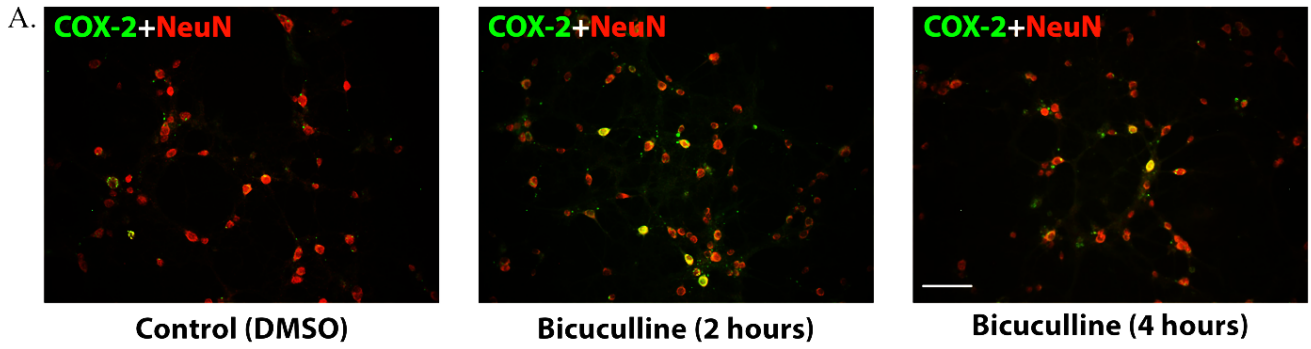
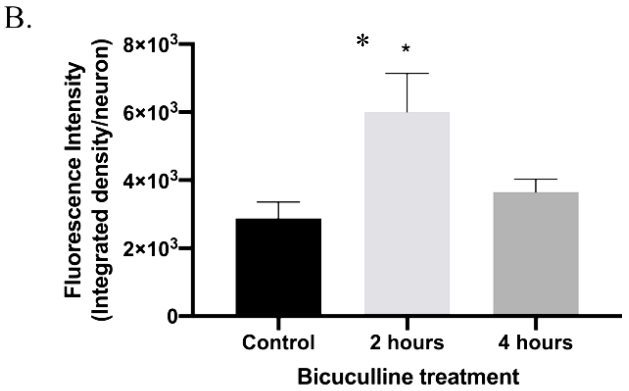


Fig. 4.11 Expression of COX-2 and excitation-coupled gene, cFos increases in hippocampal neuron cultures after Bicuculline treatment but attenuated with MK-801 treatment.

Cultures were treated with 0 (Control) or MK-801 (10 μM, 30 min pre-treatment), followed by 0 (DMSO) or Bicuculline(100μM, 60 min treatment) (N=3). Normalized mRNA levels were compared between treatments using 2-Way ANOVA followed by Uncorrected Fisher's LSD test (*, significantly different between treatments, $p < 0.001$). Annotations for this graph were used to designate individual significance between treatments and described in the following table containing individual *p* values.

Uncorrected Fisher's LSD	Significant?	Summary	Individual P Value
COX-2			
control vs. MK-801	No	Ns	0.1405
control vs. Bicuculline	Yes	*	<0.0001
control vs. MK-801+Bicuculline	No	Ns	0.5430
MK-801 vs. MK-801+Bicuculline	No	Ns	0.3667
Bicuculline vs. MK-801+Bicuculline	Yes	#	<0.0001
c-Fos			
control vs. MK-801	No	ns	0.2177
control vs. Bicuculline	Yes	•	0.0002
control vs. MK-801+Bicuculline	No	ns	0.5051
MK-801 vs. MK-801+Bicuculline	No	ns	0.5559
Bicuculline vs. MK-801+Bicuculline	Yes	Φ	<0.0001





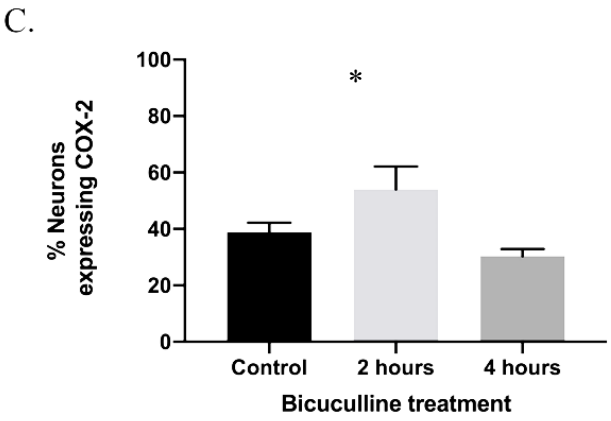


Fig.4.12. COX-2 expression is elevated with bicuculline treatment in hippocampal neurons.

A. Representative photomicrograph (20X objective) demonstrates COX-2 protein expression (green) colocalized with neuronal marker, NeuN (red) in hippocampal neuronal cells for bicuculline treatment compared to untreated neurons after 2- and 4-hours post-treatment. Scale bar = 25µm.

B. Quantification of mean COX-2 fluorescence intensity per neuron between control and bicuculline treated cells were analyzed by one-way ANOVA, (*,p=0.0293, followed by Fisher's uncorrected LSD test for multiple comparison, *, p= 0.0108, Bic (2hours) vs. Control and p= 0.502, Bic (4hours) vs. Control). The equal variance was validated by the F test.

C. Quantification of % neurons expressing COX-2 was compared between control and bicuculline treated cells. % of neurons expressing COX-2 protein by one-way ANOVA. (*,p=0.0437, followed by Fisher's uncorrected LSD test for multiple comparison, , p= 0.0658, Bic (2hours) vs. Control and p= 0.3439, Bic (4hours) vs. Control). The equal variance was validated by the F test.

4.4.6 Effect of IL-1R1 receptor neutralization on basal and induced COX-2 expression in hippocampal neurons

Both basal and neuronal activity dependent COX-2 expression were significantly lowered in hippocampus of mice with genetic deletion of IL-1R1. To test if this observation recured *in vitro*, IL-1R1 neutralizing antibody was utilized to block the IL-1R1 receptor function in cultured primary hippocampal neurons. Constitutive COX-2 immunoreactivity in distinct neuronal population of hippocampus was elevated by excitatory neuronal activity (Fig 4.12 & 4.13 C).

Basal COX-2 expression (Fig. 4.13, Panel A) was significantly lowered (52%) in hippocampal neurons following 120 mins of IL-1R1 neutralizing antibody treatment (Fig 4.13, panel B). Secondly, IL-1R1 neutralization antibody pretreatment caused significantly lower (72.5%) activity dependent COX-2 expression (Fig. 4.13, Panel D), compared to bicuculline induced COX-2 immunoreactivity in hippocampal neurons (Fig. 4.13, Panel C).

Basal and induced COX-2 expression was lower with IL-1R1 neutralization treatment *in vitro*, and this study showed corresponding outcomes as seen *in vivo* with IL-1r1 mutant mice, indicating modulation of COX-2 expression when IL-1 signaling is not functional, both *in vivo* and *in vitro*.

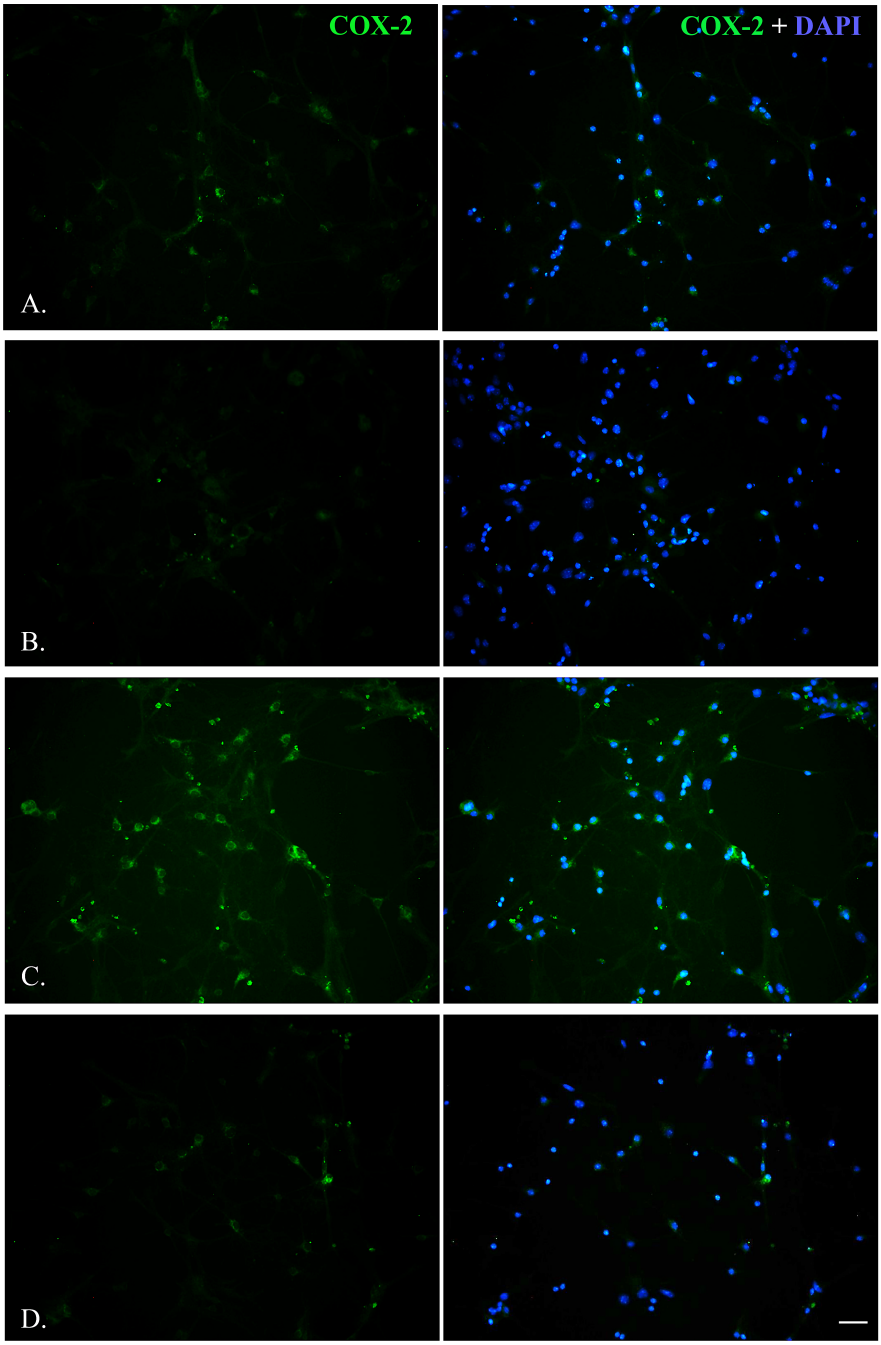
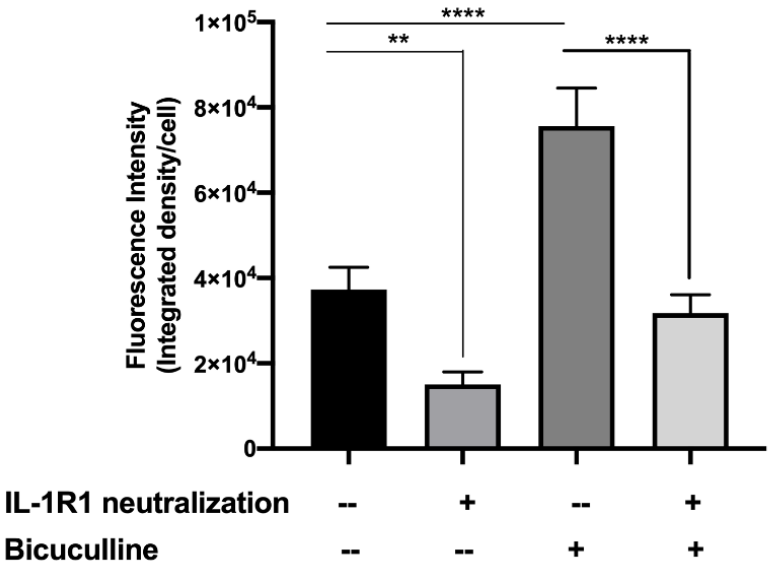


Fig.4.13. IL-1 receptor neutralization lowers constitutive and activity dependent COX-2 expression *in vitro.*

i. Representative photomicrographs of hippocampal neurons in culture with following treatments exhibited COX-2 immunoreactivity (green) co-stained with DAPI (blue) (20X objective).
A. Constitutive COX-2 immunoreactivity in hippocampal neurons, **B.** with 120 min IL-1R1 neutralizing antibody treatment, **C.** with 120 min bicuculline treatment, and **D**. 120 min pretreatment with IL-1R1 neutralizing antibody followed by 120 min of bicuculline treatment. Scale bar = 25µm.

(continued)



ii. COX-2 fluorescence intensity per neurons was quantified, compared between treatments and analyzed using Ordinary one-way ANOVA, followed by Fisher's uncorrected LSD test for multiple comparison (*, $p<0.0001$). Equal variance was validated by F test.

Uncorrected Fisher's LSD	Significant?	Summary	Individual *p*-Value
- IL-1R1 Ab v/s +IL-1R1 Ab	Yes	**	0.0089
- IL-1R1 Ab v/s +Bicuculline	Yes	****	<0.0001
+ Bicuculline v/s +IL-1R1 Ab + Bicuculline	Yes	****	<0.0001

4.4.7 Effect of IL-1R1 gene deletion on basal and induced PGE_2 level

COX-2 is the rate-limiting enzyme required for prostaglandin synthesis from arachidonic acid. Being the key enzyme of this biochemical pathway, alteration in availability of COX-2 may skew physiological and neuronal activity dependent prostaglandin production and function. To test the hypothesis whether lowered basal and induced COX-2 may also affect prostaglandin production, constitutive (naive) and intensive neuronal activity dependent (43.5mg/kg PTZ treated) PGE_2 levels were measured in wild type and mutant IL1r1 littermate mice hippocampi. PGE_2 measured via indirect ELISA demonstrated genotype and neuronal activity dependent variation (Fig. 4.14). Wild type mice hippocampi generated 130.3±3.9 pg/ml PGE_2 in basal state. However, immediately following a convulsion, the PGE_2 level increases approximately 4 times of its basal level (514±32.4 pg/ml) in the hippocampi of PTZ treated WT mice. Hippocampi of WT mice have approximately 50% lower constitutive PGE_2 level compared to their KO counterparts.

Following a convulsion, WT mice hippocampi generated 4 times higher PGE_2 than what it produced basally and 2.81 times more PGE_2 produced in the KO hippocampi following a convulsion. In contrast, the level of PGE_2 in KO hippocampi remained same after a convulsion in KO mice hippocampi (Fig.4.14). Thus, basal and induced PGE_2 production was mediated by IL-1 signaling in mice hippocampi.

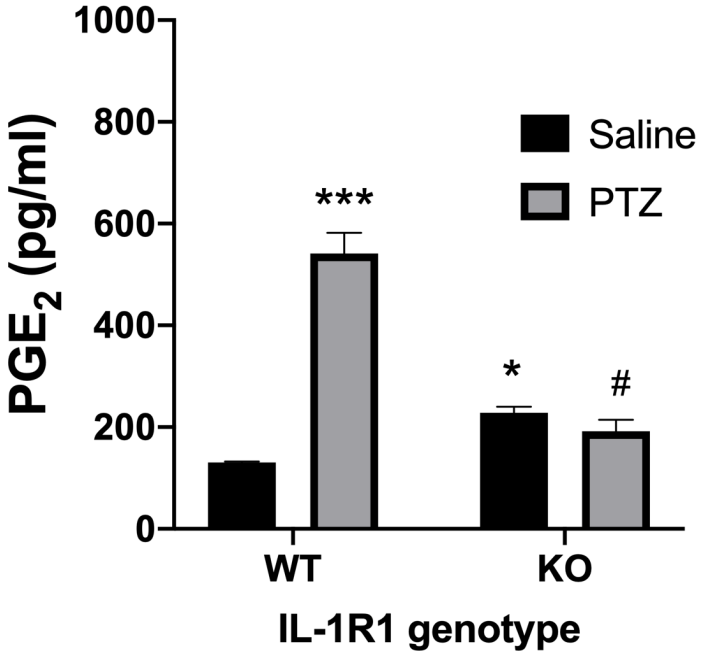


Fig.4.14. IL-1 signaling affects basal and activity-dependent PGE_2 levels in the hippocampus of mice brain.

PGE_2 levels were measured through ELISA in WT (N=3, for each treatment group) or KO (N= 3, for each treatment group) hippocampi treated with either saline or with 43.5mg/kg PTZ injection inducing convulsive seizure and analyzed using 2-way ANOVA followed by Fisher's uncorrected LSD test. Significant differences ($p<0.001$), a, between genotypes ($p=0.008$) and b, between treatments ($p<0.001$) were found. Annotations in this graph were used to designate individual significance between treatments and/or genotype and is described in the following table containing individual *p* values.

Uncorrected Fisher's LSD	Significant?	Summary	Individual *p*-Value
WT: Saline vs. WT: PTZ	Yes	***	<0.0001
WT:Saline vs. KO:Saline	Yes	*	0.0204
WT: PTZ vs. KO: PTZ	Yes	#	<0.0001
KO: Saline vs. KO: PTZ	No	ns	0.3182

4.5 Discussion

Evidence from this study supports two primary suppositions. First, excitatory neuronal activity does not alter immediate IL-1β protein levels in the normal hippocampus. Second, this report particularly draws merit in determining the role of endogenous IL-1β signaling in modulating basal and neuronal activity dependent COX-2 in hippocampus of mice brain, as role of endogenous IL-1β in mediating COX-2 have not been investigated prior to this study . Constitutive IL-1β moderates basal and neuronal activity dependent COX-2 expression and its function.

4.5.1 Effect of excitatory neuronal activity on IL-1β ligand and its signaling components.

IL-1β is a secretory protein which is produced and released primarily in cells of monocyte/macrophage lineage by stimuli either exogenous (like bacterial toxin, LPS or gamma radiation) or endogenous (pro-inflammatory cytokines) (Dinarello 2009). Endogenous IL-1β is expressed in hippocampal neurons and its physiological release is indicated to be dependent on ATP receptor P2X7R dependent, as studied in chapter 3. However, studies in previous chapter do not shed light upon the specific endogenous stimuli that may trigger IL-1β production and release from neurons.

Firstly, this study reports intensive excitatory neuronal activity (PTZ induced convulsion) causing a transient increase in IL-1β mRNA immediately following PTZ induced convulsion which recedes to basal level by 2 hours. Previous literatures have also shown induction of IL-1β gene expression due to different kinds of neuronal activity. Both *in vivo* and *in vitro*, IL-1β gene expression was elevated in the hippocampus with long term potentiation (induced at 1 hour following LTP and was highest at 3 hours post LTP in slices and 8 hours following LTP in hippocampus of rat brain) in NMDA dependent manner (Schneider et al. 1998). Fear

conditioning induced about 2.4 times more IL-1β gene expression after 24 hours in hippocampus of mice (Goshen et al. 2007) and spatial recognition memory task similarly elevated IL-1β mRNA in hippocampus but not in hypothalamus of mice within 90 min of task completion (Labrousse et al. 2009). Particularly in relevance to this result, 50mg/kg PTZ injection in rats showed similar immediate elevation in IL-1β (in 30 min) in hippocampus following PTZ induced seizure and declines by 3 hours (Minami et al. 1990). Altogether, these studies account for fact that altering excitatory neuronal activity may serve as an endogenous non-inflammatory stimulus in brain to induce IL-1β gene, specifically in hippocampus. Intensive neuronal activity did not induce either its receptor, IL-1R1 mRNA or its receptor accessory protein, IL-1RacP mRNA within first 2 hours of PTZ administration, irrespective of inducing IL-1β mRNA in hippocampus. Alteration in excitatory neuronal activity may not require IL-1R1 mRNA induction in parallel to induction of IL-1β mRNA as very few surface IL-1R1 is sufficient to elicit biological response within a cell (Bankers-Fulbright, Kalli, and McKean 1996). Endogenous IL-1R1 expression is mostly concentrated in DG neurons of hippocampus (Parnet et al. 2002; Liu et al. 2019) and may be sufficient to propagate IL-1β signals. Yet, Caspase-1 mRNA is induced at 1 hour following convulsive seizure in hippocampus of mice brain. As Caspase-1 is required for processing of inflammatory IL-1β (Pétrilli, Papin, and Tschopp 2005; Dinarello 2009), its induction may seem relevant in this scenario. As discussed in section 3.5 of chapter 3, requirement of Casapase-1 in processing endogenous IL-1β will require further investigation to understand the relevance of its mRNA induction following PTZ treatment in mice hippocampus.

It is known that endogenous IL-1β is produced, processed and released for several neuromodulatory functions in normal brain. The current study did not find any immediate change

in IL-1β ligand in hippocampus tissue following convulsion. Consistent with results in chapter 3, saline treated hippocampus showed very low basal level of IL-1β (40pg/ml). This level of IL-1β did not change following PTZ induced convulsion within an hour. It can be hypothesized that constant level of endogenous IL-1β protein in hippocampus between treatment time points as seen here may occur if excitatory neuronal activity dependent IL-1β production and release occurs simultaneously, maintaining a constant basal level of IL-1β. Alternatively, IL-1β protein may translate after 1 hour of PTZ induced convulsion. The current study differs from studies done by Temp et al. 2017, where IL-1β protein doubled its endogenous level in hippocampus of mice brain within 20 min of PTZ injection (50mg/kg, i.p.) (Temp et al. 2017).

In cultured inflammatory cells, IL-1β mRNA is stable up to 4 hours of induction before it is degraded by intrinsic mechanism (Fenton et al. 1988). Studies herein showed mRNA elevation upto 30 min before it went back to basal level by 2 hours. Therefore, a caveat in this study remains in observing IL-1β levels only for one hour. Extending the time point can suggest a different result. Further experimentation will be required to test these hypotheses.

PTZ was utilized to model intensive excitatory neuronal activity without causing excitotoxic insults in mice brain (Claycomb, Hewett, and Hewett 2011). This model is unique as it provided an opportunity to study if IL-1β is induced by alterations in neuronal activity independent of immune reactions initiated by excitoxicity as seen in kainic acid (KA) model of acute seizure. KA causes excitotoxic insults within 1-3 hours of administration (Eriksson et al. 1999) and induces IL-1β mRNA within 2.5 hours of administration (Minami et al. 1990) followed by elevated IL-1β protein by 5 hours of administration (Eriksson et al. 1999). However, IL-1β induction in this case occurs in microglia (Eriksson et al. 1999) and is associated with excitotoxic insults and neuronal cell loss (Eriksson et al. 1999; Claycomb, Hewett, and Hewett 2012)

indicating an immunological effect rather than neuronal. However, cellular site of IL-1β mRNA induction by PTZ in mice hippocampus will require further study.

Similarly, in cultured hippocampal neurons, no changes in level of IL-1β protein were observed after 4 hours of bicuculline treatment. Correspondingly, no gross differences in subcellular localization of IL-1β were observed between treatments (compared to Fig. 3.3). Furthermore, IL-1β expression in cell lysates as seen through western blot expressed only 31kD pro- IL-1β band. As IL-1β processing is associated with its release, this observation seems to be recurring occurrence across CNS cell types [studies in chapter 3 with cultured neurons with or without JNJ treatment, Fig 3.4 and in mixed glial culture (Kim, Smith, and Van Eldik 2004)]. Measuring IL-1β in cell media post treatment will be able to shed light upon constitutive production, processing and release of IL-1β induced by excitatory neuronal activity. It can be summarized herein that PTZ mediated intensive excitatory neuronal activity did not markedly elevate IL-1β protein immediately in mice hippocampus or in cultured neurons. The logical next step is to look into changes in expression or function of any effector molecules downstream of IL-1β signaling. Preliminary results on phosphorylated IRAK-1 immunoreactivity (Section 7.5, Appendix) showed, it immediately elevated following PTZ induced convulsive seizure stimuli (Fig 7.9). As IRAK-1 is phosphorylated by IRAK-4 which in turn is self-phosphorylated after its association with MyD88 adaptor protein, it can be an indication of activation of IL-1β signaling (S. Li et al. 2002; Neumann et al. 2007). To test if IL-1β signaling initiated IRAK-1 phosphorylation due to intensive neuronal activity, p-IRAK-1 immunoreactivity was tested in IL1r1 mutant mice, which showed significantly lowered p-IRAK-1 immunoreactivity (Fig 7.10 s). IRAK-1 is a key kinase to NF-kB signaling, possibly downstream of IL-1β signaling (Kishi et al. 2016). These preliminary studies indicate, firstly, IL-1β signaling may be triggered by intensive neuronal

activity (immediate phosphorylation of downstream kinase), and secondly, this phosphorylation is dependent on functional IL-1β signaling. Further studies will be required to understand the IRAK phosphorylation in this aspect in detail. Also, it can help identify IRAK-1 phosphorylation as a biomarker for acute seizure activity.

Another effector candidate of IL-1β signaling whose expression and function was investigated in detail was COX-2.

4.5.2 Effect of endogenous IL-1 signaling on basal and activity dependent COX-2 expression

COX-2 is the rate limiting enzyme in arachidonic acid pathway which produces lipid inflammatory mediators (prostaglandins) by metabolizing membrane phospholipids. COX-2 is generally induced by inflammation and its physiological expression is present only in very few specific cells of CNS including glutaminergic neurons of hippocampus (Kaufmann et al. 1996). In relevance to this report, numerous studies have identified COX-2 mRNA, protein and COX-2 dependent PG production is induced by exogenous IL-1β in CNS (Blom et al. 1997; Inoue et al. 1999; Molina-Holgado et al. 2000; Samad et al. 2001; Morioka et al. 2002; Parker et al. 2002; Hein et al. 2007; Neeb et al. 2011; Ohnishi et al. 2019b).Some studies have also shown effect of IL-1ra in moderating COX-2 (Inoue et al. 1999), however, both IL-1β and IL-1ra was provided to the system exogenously.

Endogenous COX-2 expression in neurons in CA3 region of hippocampus is lower in absence of IL-1 signaling. However, basal COX-2 mRNA levels is similar in both WT and IL-1R1 mutant mice [(Claycomb 2011) and current data] implying firstly, knocking out IL-1R1 in mice did not affect basal COX-2 transcription and secondly, absence of IL-1 signaling affected COX-2 expression in post transcriptional manner.

Post transcriptional modification in COX-2 is receiving research importance owing to its relevance in neuromodulatory roles (Hewett et al. 2016; Gong and Hewett 2018). 3'UTR region of COX-2 contains Adenylate-uridine Rich Element, which mediates post-transcriptional regulation of COX-2. No evidence as far directs towards role of endogenous IL-1β in moderating 3'UTR elements of COX-2 mRNA. However, a study done on human endometrial stromal cell show exogenous IL-1β mediated post-transcriptional modification of COX-2 mRNA by enhancing mRNA stability and sustaining COX-2 mRNA production (Tamura et al. 2002). IL-1Ra treatment on other hand lower COX-2 mRNA stability (Tamura et al. 2002). Although this study is done in different tissue type with exogenous IL-1β, this may provide clue as to how IL-1β may moderate COX-2 mRNA in hippocampal neurons and its absence (in mice with IL-1r1 deletion) can alter mRNA stability and not its production, lowering basal expression of COX-2 protein. Secondly, even though COX-2 immunoreactivity is significantly lower in mutant mice, COX-2 expression is not completely abrogated indicating parallel mechanism on which basal COX-2 production may be dependent, alongside IL-1 signaling.

PTZ induced neuronal activity mediates the following changes in COX-2 expression in mice hippocampus, i) both in wildtype and mutants, PTZ induced acute convulsive seizure induces COX-2 expression in DG subregion and elevates activity dependent COX-2 expression in CA3 subregions by 1 hour, alongside, it also induces COX-2 expression in CA1 sub-region but only in the wildtypes, ii) at three hour following PTZ injection, COX-2 expression remains elevated in CA3 and DG region, which is consistent with previous findings (Claycomb, Hewett, and Hewett 2011; Gong and Hewett 2018) and iii) Activity dependent COX-2 expression is lower in the mutant mice in CA3 and DG regions at both time points, however, is absent in CA1 region.

Firstly, role of endogenous IL-1 signaling is implied in activity dependent COX-2 expression. Like its basal expression, activity dependent COX-2 is not completely dependent on IL-1 signaling as COX-2 expression lowered but was not completely abolished. Secondly, this study demonstrates COX-2 protein induction occurs very quickly in all three regions of hippocampus in WT type however, only DG and CA3 but not in CA1 of mutant mice at a lower rate. IL-1β needs to be secreted and bind to cell membrane receptor, IL-1R1for signaling. Studies from chapter 3 have indicated endogenous IL-1β may be located in CA3/CA1 pyramidal neurons. IL-1R1 localization in hippocampus therefore should suggest cells involved in IL-1 signaling. In mice, IL-1R1 protein is shown to concentrated in the cell soma and molecular layer of DG granule cells (Parnet et al. 2002; Liu et al. 2015), however expressed throughout CA1-CA4 (French et al. 1999). Although, whether endogenous IL-1β release from neuron is synaptic or extra-synaptic, is not yet investigated, this report is indicative of an autocrine signaling mechanism within CA3/CA1 neurons and paracrine signaling in DG neurons of hippocampus via which IL-1β signaling may regulate COX-2 function.

Lastly COX-2 is induced in CA1 neurons of hippocampus of wild type mice within one hour, which completely abolishes by 3 hours post injection. COX-2 is induced in CA1 neurons of rat hippocampus in sub-convulsive electric stimulation model of kindling after 24 hours (Chen, Magee, and Bazan 2002), but no previous report mentions rapid COX-2 induction followed by rapid abolition in CA1 region in any acute seizure paradigm. However, no expression of COX-2 in CA1 neurons of mutant mice may indicate COX-2 induction in these CA1 neurons to be moderated specifically by IL-1β signaling, however, further study will be required to confirm this notion and its relevance.

Using cultured hippocampal neurons, it was once again shown endogenous COX-2 mRNA is induced by glutaminergic signaling [Fig 3.8 & (Yamagata et al. 1993; Stark and Bazan 2011)]. MK-801 treatment by itself lowered COX-2 induction however not significantly. The deficiency in effect of MK-801 by itself, which is expected to suppress expression mediated by excitatory activity, indicates that this basal inhibitory condition is sufficient to suppress to a large extent of basal excitatory activity in these cultures as seen also with APV treatment (Fig. 3.8, Chapter 3) . However, bicuculline mediated elevation in both c-Fos and COX-2 mRNA which is significantly suppressed with pretreatment with MK-801, confirms COX-2 mRNA induction to be NMDA dependent in cultured hippocampal neurons. Bicuculline induces COX-2 protein by two hours of treatment. It is utilized to model and test if COX-2 induction was IL-1 signaling dependent. Basal and activity dependent COX-2 immunoreactivity is lower with IL-1R1 neutralization in cultured hippocampal neurons which corroborates with the *in vivo* studies. Simultaneously, it indirectly confirms the source of IL-1β which moderates COX-2 expression to be neuronal. Cellular signaling mechanism connecting endogenous IL-1β and COX-2, however, will need further investigation.

4.5.3 Effect of endogenous IL-1 signaling on basal and activity dependent PGE_2 level

Prostaglandin level elevates after convulsive stimuli (Förstermann et al. 1982). Among the prostaglandins expressed in CNS, PGE2 is highly studied in CNS owing to its neuro-inflammatory roles (Lima et al. 2012). Moreover, exogenous IL-1β elevates PGE_2 in COX-2 dependent manner in CNS in rat hippocampus (Hein et al. 2007) or in cultures like murine astrocytes (Blom et al. 1997; Molina-Holgado et al. 2000), mixed glial culture (Parker et al. 2002), in neurons and astrocytic mixed culture from trigeminal ganglia (Neeb et al. 2011). It also

induces COX-2 dependent prostaglandin synthesis in neurons of spinal cord (Samad et al. 2001) and dorsal root ganglia (Inoue et al. 1999; Morioka et al. 2002).

Constitutively, there is low constitutive level of prostaglandins expressed in brain (Förstermann et al. 1982). Low level of PGE_2 in saline treated WT hippocampus which elevates immediately after convulsive stimuli is consistent with this finding (Berchtold-Kanz et al. 1981; Förstermann et al. 1982). Convulsive stimuli did not induce PGE_2 levels in IL-1r1 KO mice but did not affect the basal level. As PGE_2 was measured in hippocampus immediately after convulsion, this observation can be attributed to reduced endogenous COX-2 in the mutant mice. However, elevated level of PGE_2 in KO hippocampi can be indicative of compensatory COX-1 dependent PGE_2 production due to lowered constitutive COX-2. Exogenous IL-1β is shown to elevate both isoforms of PGE synthase (enzyme), COX-2 dependent membrane associated mPGES and COX-1 dependent cytosolic cPGES/p23 (Moore, Olschowka, and O'Banion 2004). But role of endogenous IL-1β in mediating PGESs are not known yet. As, COX-1 is basally expressed in CNS and link between endogenous IL-1β and COX-1 is not studied herein, it may be premature to comment on compensatory role of COX-1 mediated PGE_2 synthesis in IL-1R1 mutant mice hippocampus without further study.

Prostaglandins generated from convulsive stimuli have shown several protective properties against acute seizure (Förstermann et al. 1982). Further studies will be required to understand if elevated PGE_2 in WT mice following convulsion provides any neuroprotection which may be lower or absent in the mutants due to absence of PGE_2 synthesis by convulsive stimuli . To summarize, this study indicated role of endogenous IL-1 signaling in moderating endogenous and convulsive stimuli induced PGE_2 levels in COX-2 dependent manner. Thus, this report identifies PTZ induced convulsion (or intensive neuronal activity) as an endogenous non-

inflammatory stimulus to induce endogenous IL-1β mRNA in mouse hippocampus. It also reports a novel link between endogenous IL-1 signaling and COX-2 in hippocampal neurons.

Chapter 5

Development and maintenance of murine primary hippocampal neuronal culture.

5.1 Summary

Optimized cell culture systems have been applied for an array of biochemical and imaging studies to complement and model biological systems. For this dissertation, murine hippocampal neuronal cultures were cultured and maintained as a research tool. Murine hippocampal neuronal cultures were prepared and maintained utilizing a standardized protocol which includes isolating, successful culturing and upkeep of these neurons in tissue culture plates without astrocyte feeder layers from postnatal mouse pups. Alterations within established protocols were made to maintain this working culture system. This chapter will describe the standardization of the culture protocol in the Hewetts' lab and how it differed in certain aspects from already published protocols. Firstly, this chapter enlisted the changes made within the established protocol to formulate this working procedure and justified the changes made for growing neurons *in vitro*. Secondly, the study also characterized endogenous expression of key IL-1 signaling components in the murine hippocampal neuronal culture to further strengthen its relevance in context of this dissertation research.

5.2 Introduction

The detailed process of how primary hippocampal neuronal culture was developed and maintained as a working protocol in this lab will be discussed in this section. As this culture system was a new model system in our laboratory, it required development of a standardized protocol for successful culture of murine hippocampal neurons.

Prior to delving deeper into the description of the procedure for this culture system, it was important to shade light on the relevance of hippocampal neuronal culture system in lights of the dissertation. Using P2X7R antagonism, it was seen IL-1β accumulated in CA3/CA1 pyramidal

neurons of hippocampus in mice brain (Chapter 3, Fig 3.1). Based upon this result, it was hypothesized that constitutional IL-1β release is neuronal as opposed to glial IL-1β release and functioning in neuroinflammatory or neurodegenerative processes. Culturing hippocampal neurons would directly able us to test the hypothesis that *constitutive IL-1β is neuronal* without confound. Studies discussed until now (in chapter 3 & 4) have shown constitutive IL-1β to be neuronal and required for the maintenance of excitation/inhibition balance. Utilizing both *in vivo* and *in vitro* approaches, release of IL-1β from hippocampal neurons was indicated to be P2X7R mediated (studies in chapter 3). This study also paved way for a novel approach in studying in detail the sub-cellular localization of IL-1β, functional relevance of its physiological expression and release (Chapter 3 and 4) and its possible downstream signaling mechanism in neuromodulation (Chapter 4). To successfully execute these studies, *in vitro* approaches were considered alongside utilizing mice as model for investigation. Thus, the goal of this chapter was to describe in detail, a working protocol developed in this lab to culture and maintain of near-pure, primary murine hippocampal neurons which was utilized for studying role of constitutive IL-1β in neuromodulation.

This chapter will firstly describe how this protocol differs from some of the established protocols in details and secondly, describe some of the characterization studies done to rationalize the potential of this culture system to study role of constitutive IL-1β release and functioning in neuronal excitation.

5.3 Culturing murine Hippocampal Neurons

Cells *in vitro* requires specific media for its culture and growth depending on its cell and tissue types, origin and methods of culture. For culturing hippocampal neurons, initially, all media and

reagents were prepared as described in (Beaudoin et al. 2012) and was applied to the culture system, however, it did not yield viable neurons in culture. After reviewing multiple research protocols for this culture system, procedures from few protocols were combined to yield viable hippocampal neurons *in vitro*. Hippocampal neuronal cultures were prepared from postnatal day 0-1 CD-1 mouse pups using a protocol established from several previous studies (Ma et al. 2003; Uliasz et al. 2012; Beaudoin et al. 2012). The following section will broadly describe the changes and alterations made in these studies.

5.3.1 Materials

5.3.1.1 Mice: Post-natal day 0 (P0) or post-natal day 1(P1) pups were taken from time pregnant CD-1 mice (Charles River Laboratories).

5.3.1.2 Reagents for culturing neurons: are described in detail in table 1.4 of chapter 1.

5.3.1.3 Preparation of media and reagents

Changes made into the media preparation for the current procedure is enlisted within the report. The plating and growth media were prepared following Ma et al. 2003, the dissection media was prepared following Uliasz et al. 2012.

- **10X Dissection Media (DM)-** 10X dissection media was made as described in (Uliasz et al. 2012). In short, 25 g glucose, 35 g sucrose, and 24 g HEPES was diluted in 500 ml 10X Hank's Balanced Salt Solution (HBSS) without calcium and magnesium and pH was adjusted to 7.4 and filtered through a 0.22µM cellulose nitrate filter system (Corning) for sterilization and stored at room temperature of 4°C. 1X DM was used during dissection and was made by diluting it in sterile water 1:10 and stored at 4°C.
- **Hippocampal Plating Media A (PMA)** used for this culture was prepared following (Ma et al. 2003)**.** In short, 7% v/v heat inactivated Horse Serum, 1% v/v B27 Supplement

(50x) , 5 µM β-mercaptoethanol (2-ME), 0.5mM glutamine, 10ml/l Penicillin/ streptomycin (100x, final conc.) were dissolved in Neurobasal A Medium. 100-200ml media were made, sterile-filtered through 0.22µm cellulose nitrate filter system (Corning) and stored in 4ºC for up to 2-3 weeks. The primary difference from already established protocol referred above was the use of lower concentration of 2-ME (5µM) compared to 25µM mentioned in Ma et al. 2003.

- **Hippocampal Plating Media B (PMB)** used for this culture was prepared following (Ma et al. 2003)**.** Briefly, 3% v/v heat inactivated Horse Serum, 1% v/v B27 Supplement (50x), 5µM 2-ME, 0.5mM glutamine, 1% v/v Penicillin/streptomycin (100x) were dissolved in Neurobasal A Medium. 100-200ml media were made, sterile-filtered through 0.22µm cellulose nitrate filter system (Corning) and stored in 4ºC for up to 2-3 weeks.
- **Maintenance Media (MM)** used for this culture was prepared following (Ma et al. 2003)**.** 2% B-27 (50x), 5µM 2-ME, 0.5mM glutamine, 10ml/l Penicillin/streptomycin (100x) were dissolved in Neurobasal A medium. 300ml media were prepared, filtered through a 0.22µm cellulose nitrate filter system (Corning) and was stored in 4ºC for 2-3 weeks.
- **Ara-C (Cytosine Arabinoside)** used for this culture was prepared following (Uliasz et al. 2012)**.** In short, 20 mg Cytosine Arabino-furanoside were dissolved in 125 ml sterile Mediatech Cellgro H_2O, sterile filtered through 0.22µM cellulose nitrate filter system (Corning) to make 8µM stock of Ara-C and stored as 1 ml aliquots in -20°C. For this culture system, 6µM Ara-C was used by diluting it in MM.

5.3.1.4 Coating culture plates

Initially, cultures plates were lined with Poly-D-Lysine (PDL) coated coverslips (Neuvitro, Cat# GC-12-PDL) – 12 mm diameter (can be stored in -20°C for 6 months) (Ma et al. 2003). Sterile forceps were used to place one coverslip/well 1 hour before plating cells. Due to variation in viability of cells observed in cells plated on different batches of PDL coated coverslips, culture plate coating were switched to Poly-L-lysine following the protocol described by (Beaudoin et al. 2012). In short, to make 100µg/ml stock solution of PLL, the bottle containing 5mg of PLL is brought to room temperature by keeping it in room temperature for 30 min and dissolved in 50 ml borate buffer (made with 1.24g boric acid and 1.9g Sodium tetraborate dissolved in 400ml of sterile H_2O, maintained to pH 8.5 and sterile filtered through 0.22µM cellulose nitrate filter system). The bottle was left undisturbed for 40min-1 hour to ensure PLL had dissolved completely in borate buffer. It was mixed well prior to aliquoting and stored at -20°C. To coat the plates, on the day of dissection, 100 µg/ml stock solution (thawed) was dissolved in borate buffer in a 3:7 ratio to obtain a final concentration of 30µg/ml**.** Culture plates were coated at room temperature for at least 1 hour prior to dissection. The unbound PLL solution were aspirated, plates were washed thoroughly with cell culture grade water (3 times) and allowed to air-dry in the hood with plate lids off (dried at least for one hour).

5.3.2 Mouse pup brain dissection for hippocampal neuronal culture

Hippocampal neuronal cultures were prepared from the hippocampus of postnatal pups (P0/P1 days old). Equipment were set up before dissection**,** sterile euthanasia of postnatal pups and isolating pup brains for dissection were done primarily following (Uliasz et al. 2012)**.** For each set of hippocampal dissection, (1 set of dissection yielded cells to be plated in one 24 well cell

culture plate/one 6 well culture plate) 8-9 pups were euthanized, and their brains dissected for obtaining hippocampi.

The pup brains were isolated after setting up the dissection tools in sterile manner as follows: Four #3C Dumont Forceps, one #4 Dumont Forceps, one Rat Tooth stainless-steel Forceps (1 x 2 teeth), one curved Metzenbaum Dissecting Surgical Scissor (5 ½"L) were placed in 70% ethanol and soaked for $\geq$ 15 min to be used for dissection. Parallelly, the dissecting hood was wiped with 70% ethanol and the airflow system was turned on approx. 15-20 min prior to use. Two 150 mm Petri dish bottoms (to act as ice container) and four 150 mm Petri dish lids (tool and dish trays) were wiped with 70% ethanol and placed in the dissecting hood. Ice was placed on both 150mm dish bottoms, one of them covered with 150 mm lid over one plate of ice to act as a tray for media dishes. The other plate of ice was placed on the dissecting scope stage. Ethanol-soaked tools were carefully laid out on the sterile pads on the petri dish inside dissection hood. Four 60 mm dishes with 3ml and one 35 mm dish with 1 ml DM were kept on the ice tray to avoid cellular degradation of the dissected tissue. 2.5 ml-3 ml dissection media containing final concentration of 0.25% trypsin was prepared and kept on ice to dissociate the dissected hippocampal tissue. Once the set-up was complete, 8 pups/dissection were euthanized, decapitated and their heads were cleaned in ethanol and in dissection media following (Uliasz et al. 2012).

To dissect hippocampi from pup brain, visual guides were used as described in (Seibenhener and Wooten 2012; Beaudoin et al. 2012).The dish containing sterile heads was then placed in the center of the ice bath on the dissecting scope stage are brains were teased out of the skull using two Durmont #3C forceps after the skin from the top of the skull were carefully peeled away, while remaining cognizant of the entire dissection process is being done in cold temperature to

avoid tissue disintegration. All of the brains were transferred to a new clean dissection media containing dish. Next, using fresh set of Durmont #3C forceps, the brains were placed with dorsal side up (under 2 to 2.5X magnification) and carefully the cortex from the left hemisphere was flipped open as described in (Seibenhener and Wooten 2012; Beaudoin et al. 2012). Once the hippocampus was identified under the dissecting scope, it was slowly separated from the cortex and the meninges on top of the hippocampus were removed very carefully without disturbing or cutting through the hippocampus in the process. Once the meninges were removed, the hippocampus was removed by snipping it along the edges. The isolated hippocampus was then transferred to a 35 mm dish containing DM. Similarly, the hippocampus from the right hemisphere was also dissected. Once 16-18 hippocampi are dissected out, they were minced using Durmont #4 forceps and transferred to the dissection media containing trypsin (3 ml) and incubated for 12 min. at 37°C (water bath).

5.3.3 Plating and growing neurons in culture

Neurons were plated as described by (Beaudoin et al. 2012). However, the procedure published by Beaudoin et al. primarily varied from the current technique used are in usage of different media to plate and grow neurons (which were prepared following Ma et al. 2003), different timing for which cells were incubated in those media (designed after few rounds of problem solving to yield viable neurons) and in few key plating techniques as described below.

Plating medium A was warmed to 37°C parallelly (10 ml/ plate was required, therefore ~12 ml was warmed) while the hippocampal tissue was trypsinized. For triturating the cells, in the laminar flow hood, a flame was used to reduce the bore size of one cotton plugged pipette to ~3/4 of normal (medium bore) and another to ~1/2 of normal (small bore). Rubber bulbs were

attached, and the set-up was set aside on a pipette rack with tips up as described in (Beaudoin et al. 2012).

After the tissue in trypsin containing DM is incubated for 12 min in 37°C, the tube containing hippocampal tissue was removed from the water bath and spun in a centrifuge at low speed for 3 min (700xg). This step differed from the original protocol which allotted wait time for tissues to settle and not centrifuge. The current study however accounted for the same total time in trypsin containing media (15 min). Post centrifugation, the dissociated hippocampal tissue pieces which settled at the bottom of the tube was collected using the regular bore pipette into a 15 ml tube containing 1.5 ml of plating medium A in the tissue culture hood and triturated for 4-6 times. Next, using the medium bore pipette, the tissue was gently triturated 5-7 times to suspend the neurons. Lastly, it was triturated 5-6 times with the small-bore pipette. Although, the overall technique was adopted from Beaudoin et al. 2012, 15 ml tube were used instead of petri dish to ensure minimal surface area for the process and to avoid formation of bubbles in the media while trituration. Number of times the tissue was triturated were also reduced to avoid excessive cell breakage.

After waiting for 30-40 seconds for the bigger visible tissue debris to settle, the cell containing media was pipetted and transferred to a new tube containing about 1.5 ml plating medium, swirled gently to mix and the volume made up to 5ml without forming bubbles.

Approximately 300,000 cells/ml can be obtained in plating media (standard cell counting procedure using hemocytometer and Trypan blue staining as described in (Beaudoin et al. 2012) and was done initially to plate similar number of cells/plate. Prior to plating the cells, 200µl of plating media A is added to each well for 24 well plates (or 800µl for 6 well plates). Next, 200µl of cell suspension were added to each well for 24 well plates (or 800µl for 6 well plates). Plates

were then covered and placed in a humidified incubator with 5%-6% CO_2/air at 37°C. (Few hours after the cells were plated, to check if cells have attached to plates, the plates were swirled gently a couple of times under the hood and immediately observed under the scope to see if any cells were still floating. The media were not switched if cells were not attached to the bottom of the plate and still floating).

After 12-16 hours of plating, plating media B was warmed to 37°C and the cells are switched from PMA to PMB. However, the technique to switch the media were different from Beaudoin et al. 2012, which aspirated the entire media gently prior to adding new media. To ensure the cell layer were never exposed to air to avoid oxidative damage, in this current method, 200µl/well new media were added (for 24 well plates or 800µl/well for 6 well plates), swirled gently for 1-2 times to ensure any settled debris to float and then 400µl media were removed from the well (or 1.6ml for 6 well plates). 200µl fresh media/well (or 800µl for 6 well plates) were immediately added. Longer exposure (12-16 hours compared to 4-6 hours in PMA as described in Beaudoin et al. 2012) in media ensured no sudden changes in cell environment and longer exposure to serum for their growth.

The cells were then switched to maintenance media 6-8 hours later (warmed to 37°C before use) using the above-mentioned media changing procedure. The procedure to use two plating media with different serum concentrations, prior to use of maintenance media was followed as described by (Ma et al. 2003) and alterations in their exposure time was determined after few trails which differed from one plating media followed by growth media described in Beaudoin et al. 2012. The current technique had two crucial advantages- firstly, two plating media containing 7% horse serum (PMA) followed by 3% (PMB), ensured gradual decrease in concentration of serum, thereby ensuring no rapid changes in the cell environment. Secondly, longer exposure to

serum containing media allowed cell growth without disturbances and showed better viability and cell growth compared to exposure of shorter duration.

Cells were treated with Ara-C (**6µM** final concentration) on DIV 3 for an incubation duration of 3 days, after which the media is changed and was replaced with fresh MM. Cells were fed every three days by replacing 50% of the maintenance medium with fresh medium. The growth of the cells was observed daily under the scope. Cells for experiments were typically used between DIV14/15.

5.3.4 Troubleshooting and optimization of Hippocampal neuronal culture

Noticeable problems, their possible reasons and solutions faced during culturing murine hippocampal neurons were discussed by Beaudoin et al. 2012. Troubleshooting section, in detail, discussed commonly faced problems and how to avoid these difficulties (or their possible solutions). While working on this current protocol, all relevant points from that section were taken into consideration and carefully taken care of. In this section, few more areas of troubleshooting and their process of optimization (if applicable) have been discussed.

5.3.4.1 Use of P_0-P_1 pups

On contrary to the use of E15 fetal pups for dissection of cortical neurons, hippocampal neurons are cultured from E18.5 to P_5 (Kaech and Banker 2006; Nunez 2008; Kaar, Morley, and Rae 2017). Current study utilized P0-P1 pups. Utilizing neonatal pups were beneficial than using fetal pups yielded benefit for the following reasons: firstly, the female could then be further bred to generate more pups. Secondly, hippocampal neuronal development coincides with late fetal-early neonatal period indicating that neonatal pups would likely yield more neurons than fetal pups. These were some advantages of utilizing neonatal pups (Beaudoin et al. 2012).

One study utilized P_2-P_5 pups to generate hippocampal neurons successfully and characterized them (Kaar et al. 2017). In my hands, this process was not successful, although initially the neurons from two cultures progressed similarly (Fig.5.1 A). Neurons from P2-P3 pups did not extend axons and dendrites to a similar extent compared to neurons cultured from P0-P1 pups with progression in days *in vitro* (Fig. 5.1 B & C). Also, with age, there were much higher glial contamination even after similar treatment of Ara-C [consistent with findings from (Beaudoin et al. 2012), troubleshooting section].

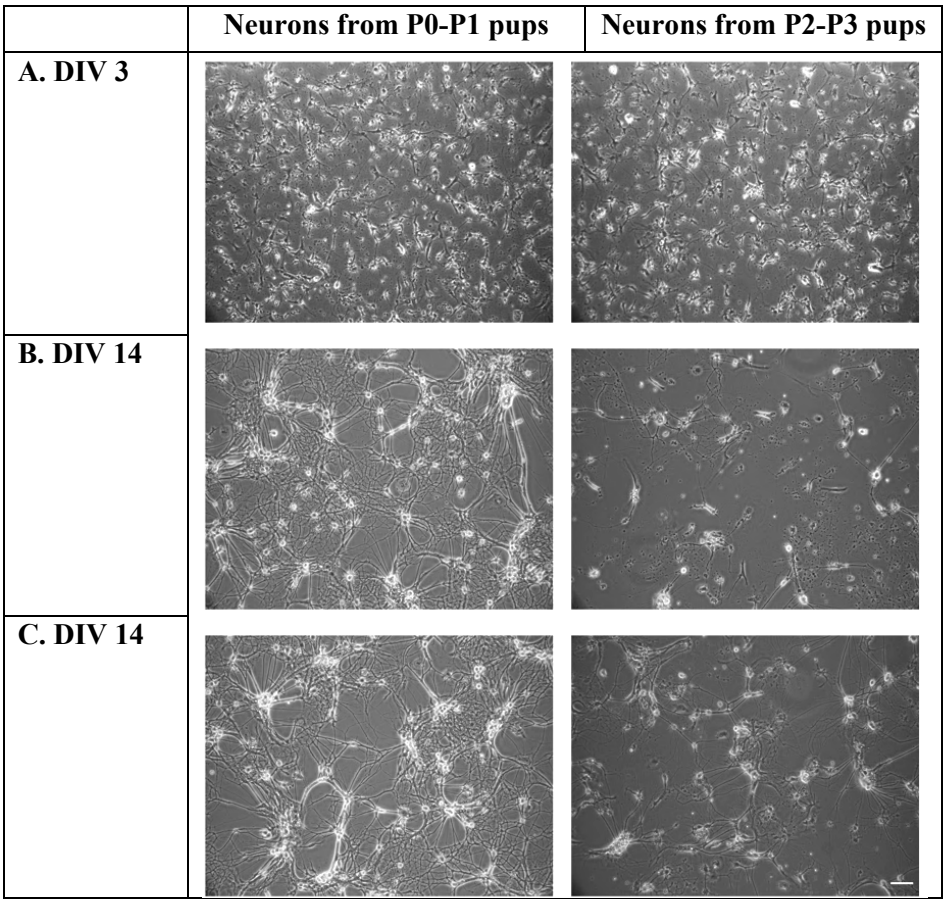


Fig.5.1. Difference in progression between hippocampal neurons cultured from P0-P1 pups v/s P2-P3 pups.

Hippocampal neuronal cell culture (10X objective) *in vitro* in DIC images demonstrated **A.** progression between neurons cultured from P0-P1pups (left) v/s P2-P3 pups (right) on DIV 3, and **B** & **C**. at DIV 14, neurons from P0-P1 pups (on left) and from P2-P3 pus (on right). Scale bar = 50 µm.

5.3.4.2 Use of Neurobasal A media

Most culture protocols utilize Neurobasal media or MEM eagle's with Earle's BSS for plating and maintaining hippocampal neurons (Beaudoin et al. 2012; Seibenhener and Wooten 2012). Initial studies utilized Neurobasal media for making growth media for the cells (Beaudoin et al. 2012). However, we switched to the Neurobasal A medium used in (Ma et al. 2008) as plating and growth media were made following (Ma et al. 2003; Ma et al. 2008). Although no parallel tests were run comparing Neurobasal Media with Neurobasal A media, according to Thermo Fisher Scientific research resource page, the difference lied in the concentration of NaCl, (4000mg/L in Neurobasal A media v/s 3000mg/L in Neurobasal media) which changed the osmolarity of the media to 260±10 mOsm.

5.3.4.3 Use of different coating media/pH

Different studies utilize different plate coating media. For ICC studies, using coated glass coverslips are a standard process. Such commercially available coated coverslips can be used, however, variations in coating between batches may exist. Initially, the present protocol utilized commercially available PDL coated coverslips to grow hippocampal neurons. However, few batches of cells died within hours of plating. When coating media was changed from PDL to PLL and rest of the procedure was kept consistent, these sudden deaths of cells were avoided. Secondly, the pH of coating media became very important factor for cell viability. Diluting PLL or PDL in sterile H_2O as suggested by manufacturer may work for other types of adherent cell types but failed to grow hippocampal neurons in culture. Diluting PLL in a slightly basic buffer [borate buffer pH 8.5 used for the studies (Beaudoin et al. 2012)] kept cells viable, hence borate buffer was used to dilute PLL for coating plates for neurons to attach and grow in culture.

5.3.4.4 Use of Ara-C

As mentioned earlier, hippocampal neurons were typically cultured from either E18.5 days or P0-P1 pups. One disadvantage of this system is the development period of hippocampal neurons coincides with the development of glial cells, unlike cortical neurons which develop earlier (Beaudoin et al. 2012). Thus, there needs to be a fine balance to treat hippocampal neurons in culture in such a period of development that it restricts glial cell growth and division without hurting neuronal cell development in culture. As primary neurons are very delicate cells in culture, care was taken to determine the right time to treat the cells with Ara-C (Beaudoin et al. 2012), which would restrict glial cell division and its growth, eventually making the culture a near-pure neuronal cell culture. Following (Beaudoin et al. 2012), we initially cultured neurons with 3 μM Ara-C on DIV-2, which initially restricted astrocyte growth as seen in Fig. 5.2 A. DIV 7 cell culture showed no astrocytes in the culture (undetectable GFAP immunoreactivity) however, with maturity, by DIV 14, the culture system showed substantial number of astrocytes in the cell culture (Fig.5.2 B) and the glial number increased with progression of days *in vitro* (Fig.5.2 C) as seen through increase in GFAP positive cells. As Ara-C was washed from the media on DIV 4 [following (Beaudoin et al. 2012)], astrocytes which were still viable left in those culture, rapidly grew and proliferated without Ara-C in media and contaminated pure neuron culture.

Glial cell growth was controlled using a higher concentration of Ara-C (6 μM). This restricted glial cell growth as seen in Fig.5.2 D and generated a near pure hippocampal neuronal culture with <10% glial cell contamination (Fig.5.6). We still observed microglia in the culture; however, we cannot treat neuronal culture with LME to eliminate microglia as it will be toxic to neurons and kill them.

Alongside transitioning from usage of two plating medias (Ma et al. 2003) from use of one plating media (Beaudoin et al. 2012), we also delayed the treatment of Ara-C from DIV 2 to DIV 3 and from 2 days of Ara-C treatment to 3 days of Ara-C treatment. Cultures were replenished with fresh media on DIV 6. This process ensured healthy growth of neurons without glial contamination in culture.

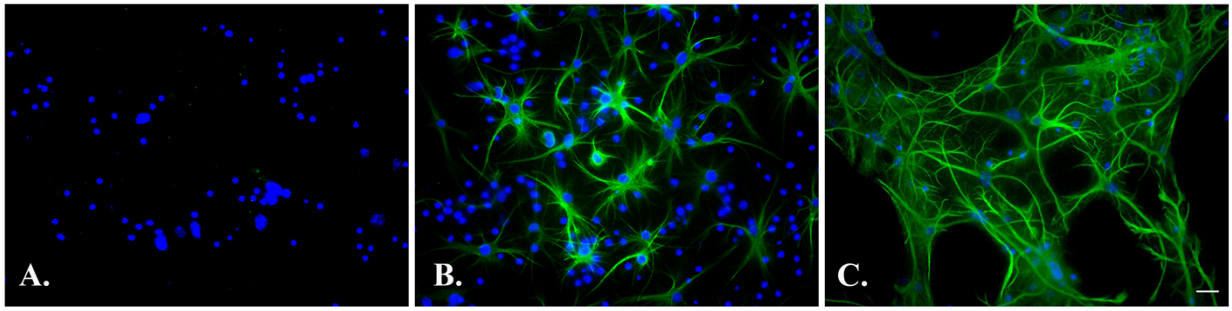
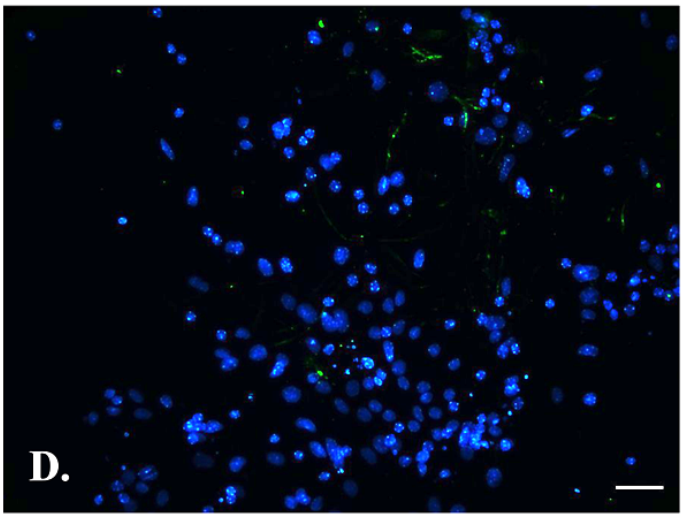


Fig.5.2. Glial contamination in culture increases with DIV with low Ara-C concentration.

Hippocampal neurons in culture showing GFAP (green) immunoreactivity in cells counterstained with DAPI at **A**. DIV 7, **B**. DIV 14, and **C.** DIV 21 (20X magnification) after being treated with 3µM Ara-C. Scale bar = 25µm.



D. Glial contamination in culture is lowered with revised Ara-C treatment.

Hippocampal neurons at DIV 14 in culture showing GFAP (green) immunoreactivity in cells counterstained with DAPI (20X objective) when treated with 6µM Ara-C for 3 days. Scale bar = 25µm.

5.3.4.5 Use of β-mercaptoethanol (2-ME)

Protocol by Beaudoin et al 2012 did not use 2-ME to culture hippocampal neurons (Beaudoin et al. 2012). However, following Ma et al 2003, using 2-ME in all of the mediums increased the survival rate and viability of the cells in the culture (Ma et al. 2003). 2-ME has been widely used for culturing neurons and its benefits in maintenance of the culture for several days are reviewed in (Ishii et al. 1993). However, the dosage of 2-ME should be tested prior to the culturing of neurons. Although the recommended dosage for culturing neurons is 10-50μM (Ishii et al. 1993; Ma et al. 2003), for my study the dosage of 2.5-5μM was adequate to maintain a healthy culture system.

5.4 Characterization of cultured hippocampal neurons

The culture of hippocampal neurons is widely used and hence, well characterized. Neurons in culture undergo distinct stages of development, starting with extension of neuronal processes, followed by specification of axon and dendrite alongside their extension. Synapse formation and maturation follows with progression of days *in vitro*. This system was also characterized to understand if the neuronal growth and development occurred similar to cultured neurons developed via previously established protocol. Herein, endogenous expression of key IL-1 release and signaling components in cultured murine neurons were also looked into, to justify its use for this dissertation research.

5.4.1 Methods

5.4.1.1 Microscopy

Live cells were imaged for monitoring growth using an inverted IX50 Olympus microscope equipped with Olympus D73 digital camera, and CellSens Standard software. Care was taken to

monitor sterility when cultured plates are taken out and into the incubator, before and after imaging.

5.4.1.2 Immunocytochemistry

Described in section 3.3.5 of chapter 3.

5.4.1.2.1 Antibodies:

Primary antibodies: **NeuN, β-III-tubulin, Synaptophysin** (Rabbit anti-Mouse), **PSD-95** (Goat Anti-Mouse), **PROX-1, GAD65/67, GABAa/α1, GFAP, P2X7R, IL-1R1, IL-1RacP, MyD88, Synaptophysin** (mouse monoclonal used for counterstaining experiment with IL-1RacP) and **PSD-95** (Mouse monoclonal used for counterstaining experiments with MyD88).

Secondary antibodies: Donkey anti Rabbit Alexa Fluor 488, Donkey anti-Mouse Alexa Fluor 488, Donkey Anti-mouse Alexa Fluor 594, Donkey Anti Goat Alexa Fluor 594, Goat anti-Rat DyLight 549, Goat anti Hamster DyLight 405. Details of antibodies are provided in table 1.3.

5.4.1.2.2 Microscopy: Images from standard epifluorescence microscopy were captured using an inverted IX50 Olympus microscope equipped with an X-Cite 120Q fluorescence light source (Lumen Dynamics), Olympus D73 digital camera, and CellSens Standard software. For high-resolution analyses, images were acquired using Zeiss Axio Observer Z1 inverted microscope equipped with 40X/1.30 oil Plan-Neofluar objective illumination using HAL100 12V 100W halogen lamp housed with microscope power supply (PhotoFluor LM-75, 89 North) and Hamamatsu CMOS ORCA-Flash 4.0 LT CCD camera (C11440-42U30). For comparisons, images from individual studies were processed identically using Adobe Photoshop.

5.4.2 Results

5.4.2.1 Days *in vitro* (DIV) characterization

The growth of hippocampal neurons in culture under this protocol were observed and matched with observation noted in anticipated result section of (Beaudoin et al. 2012).

Within DIV 2, cultured neurons started developing lamellipodia, which increased in length with progression of days in culture gradually initiating connections with other cells and processes. By DIV 7, neuronal processes formed a well-developed network with other neurons. By DIV 10, the culture demonstrated well-formed and mature neurons with structured web of the neuronal processes which has developed complex network with each other with time. Post DIV 17, the neurons formed tight aggregates or clusters connected through a tightly formed bridge of neuronal processes. By DIV 21, within some of the wells, empty spaces with no cells were noticed. Alongside loosening of the attachment caused floating of the cell bed from the sides of the well. Cell growth as observed with the current protocol (Fig. 5.3) was consistent as described in the literature (Beaudoin et al. 2012). Alongside observing live culture to understand development of neurons in culture, immunocytochemical staining with specific neuronal markers were performed to demonstrate neuronal growth and maturity.

β-III-tubulin, a neuron-specific intermediate filament, is present in the cytoskeletal structure of neuronal processes. Cultured cells were stained for β-III-tubulin immunoreactivity. As seen in Fig. 5.4, β-III-tubulin immunoreactivity allowed study the gradual growth and complexity of neuronal processes *in vitro* and validated the maturity of neurons.

Likewise, as neurons mature and develop, synapses are formed for functional neurotransmitter release and developing synaptic connections between neurons, expression of specific proteins gradually increase in neuronal synapses. As seen through Fig. 5.5, the immunoreactivity of

Synaptophysin (left column- marker for the synaptic terminal) and PSD-95 (right column- marker for post synaptic region) gradually increased through days *in vitro.* Fig. 5.4 and 5.5 thus confirmed through use of these neuronal markers that neuronal maturity is attained by DIV 10. All studies with primary hippocampal neuron cultures (studies in chapter 3 & 4) were performed at 14-15 days *in vitro* (DIV).

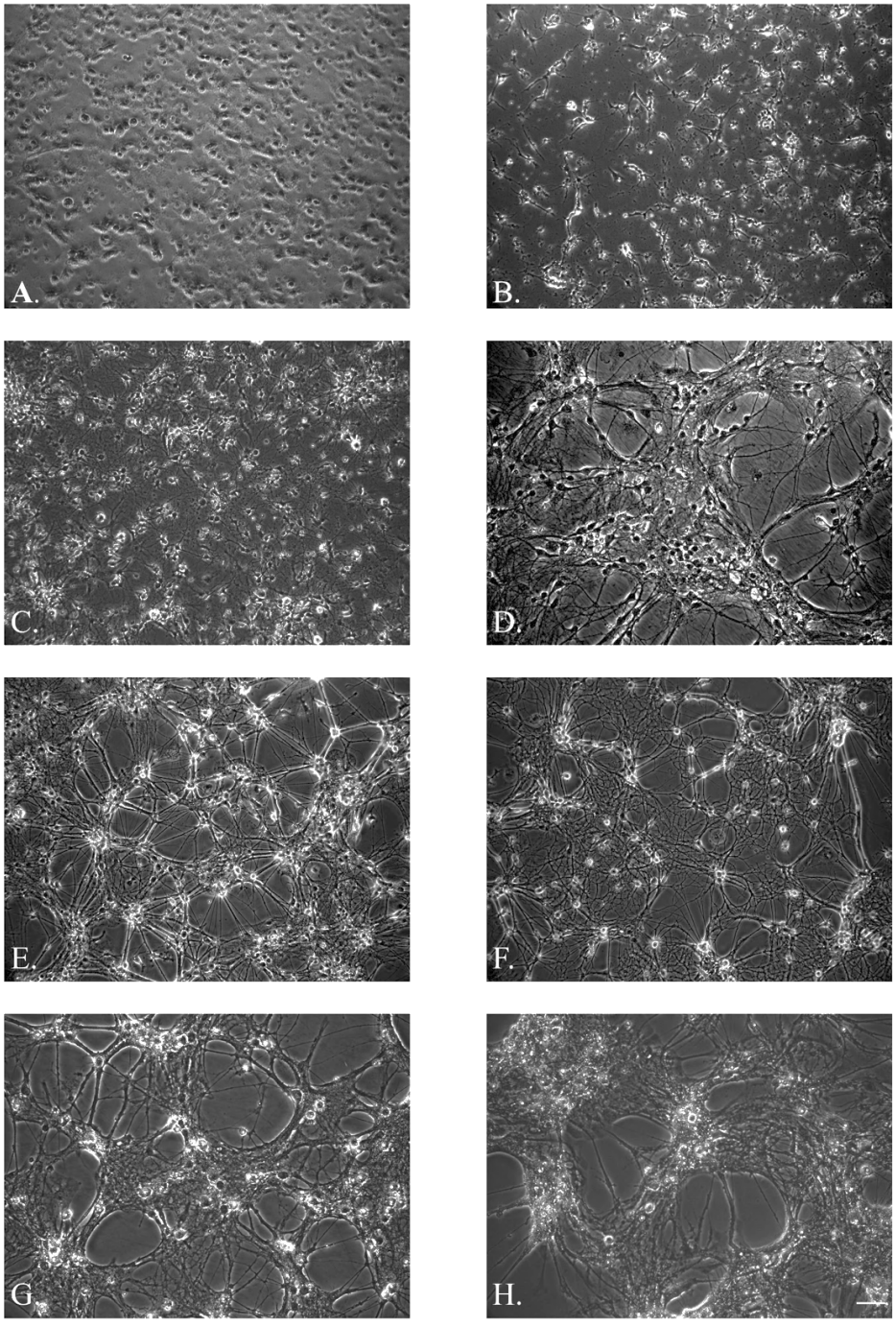


Fig. 5.3. Development of near pure hippocampal neurons grown on PLL coated culture plates.

Representative photomicrographs of cultured hippocampal neurons at **A.** DIV 2, **B.** DIV 3, **C.** DIV 5, **D.** DIV 7, **E.** DIV 10, **H.** DIV 14, **G**. DIV17, and **H.** DIV 21 demonstrated an increasing order of complexity with growth and maturity of hippocampal neurons in culture (10X objective). Scale bar = 50µm.

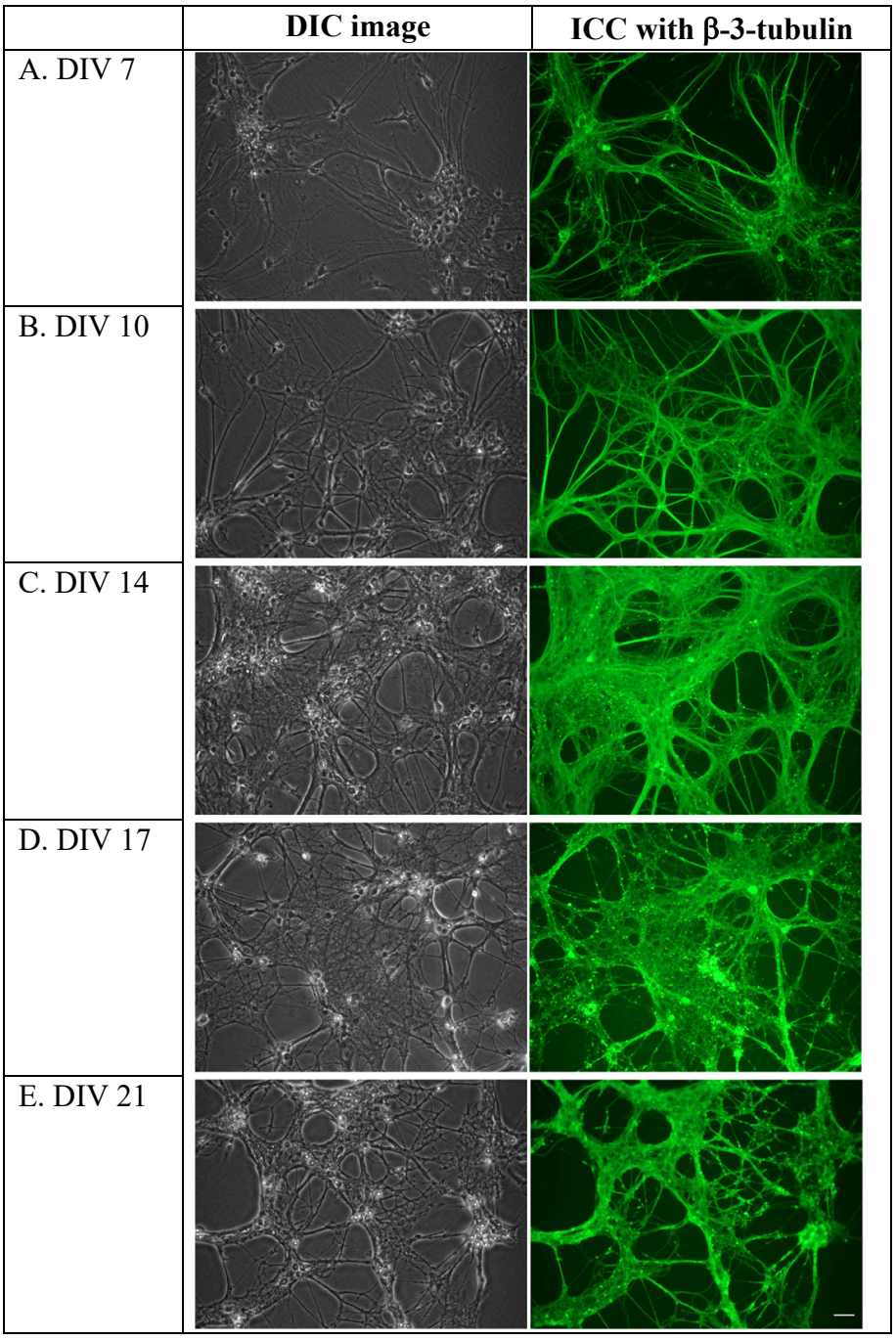


Fig.5.4. Increasing complexity of β-III-tubulin immunoreactivity in cultured hippocampal neurons with progression of days *in vitro*.

Panel (A-E) are representative photomicrographs of hippocampal neuronal cell culture *in vitro*, DIC images (on left) with β-III-tubulin immunoreactivity (in green) demonstrated increasing order of complexity with progression of days *in vitro* (10X objective). Scale bar = 25µm.

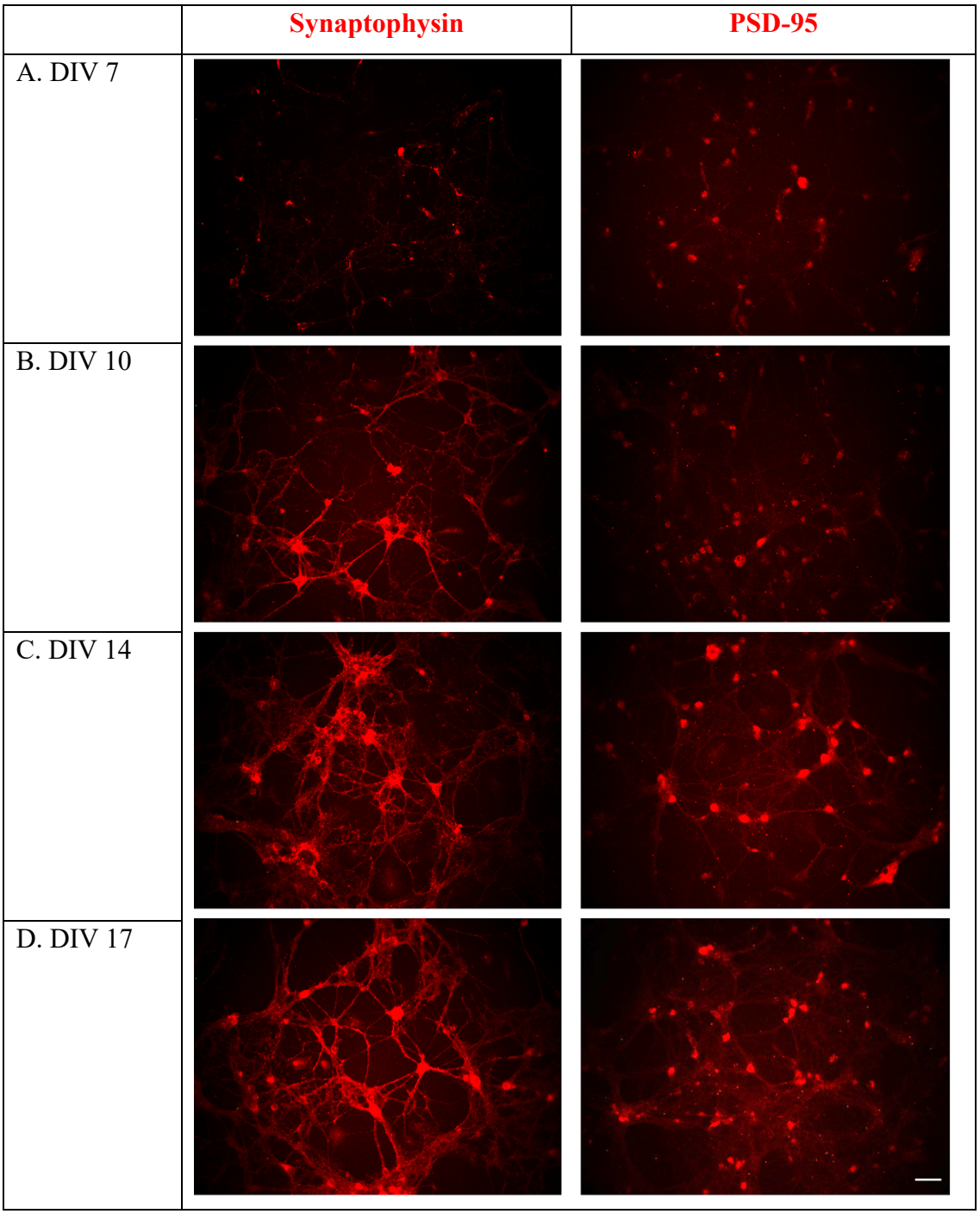


Fig. 5.5. Increasing Synaptophysin and PSD-95 immunoreactivity in cultured hippocampal neurons with progression with days *in vitro*.

Panel (A-D) are representative photomicrographs of hippocampal neuronal cell culture *in vitro*, with neuronal cell culture demonstrating immunoreactivity of pre-synaptic marker, Synaptophysin (on left) and post-synaptic marker, PSD-95 (20X objective). Scale bar = 25µm.

5.4.2.2 Culturing near pure hippocampal neurons

Cell cultures were stained for NeuN immunoreactivity (neuronal marker) and counterstained with DAPI (which stains every nucleated cell) and using cell counter plugin in FIJI, cells stained with NeuN and DAPI were counted across microscopic fields. Result demonstrated >90% of the total cells stained with DAPI also showed NeuN immunoreactivity, indicating this culture to be near pure neuronal culture. This result is consistent with neurons cultured through protocol discussed by Beaudoin et al. 2012. Fig. 5.6 demonstrates cell culture with NeuN staining counterstained with DAPI (DIV 14). As shown in the right panel marked with white arrowhead, there are presence of few non-neuronal cells.

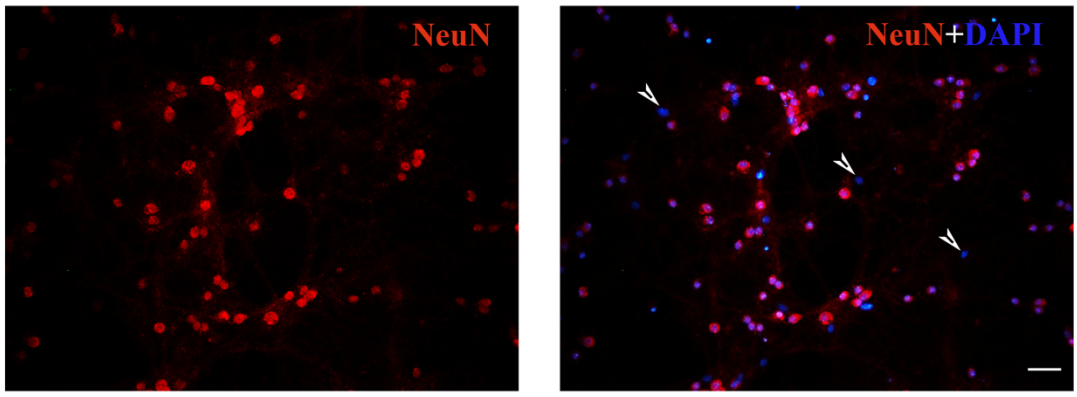


Fig. 5.6. Primary hippocampal neuronal culture may contain few non-neuronal cell types.

Representative photomicrographs of hippocampal neuronal culture (DIV 14) stained with neuronal marker NeuN (red, left panel) counterstained with DAPI(blue, right panel) (20X objective). Scale bar = 25µm. White arrowheads indicate the non-neuronal cells only stained blue on the right panel.

5.4.2.3 Certain population of hippocampal neurons are inhibitory in nature

Previously published protocols identified majority of the neurons in these preparations as excitatory neurons, with inhibitory neurons representing about 18–20% of these population. GAD 65/67 immunoreactivity is commonly used to identify inhibitory neurons. Hippocampal culture maintained using the current protocol similarly showed fairly similar percentage of

inhibitory neurons (Fig. 5.5). GABAa/α1 is another commonly utilized marker of inhibitory hippocampal neurons of all sub-regions [Hippocampome.org, (Wheeler et al. 2015; Hamilton et al. 2017)]. Culture established with the present protocol also showed a heterogeneous distribution of neurons expressing GABAa/α1(Fig 5.6). c-Fos levels measured with APV or MK-801 treatment (chapter 3 and 4) indicated strong basal $GABA_A$ receptor-mediated inhibition in hippocampal neuron cultures, possibly mediated by these GAD 65/67 positive neurons in the culture.

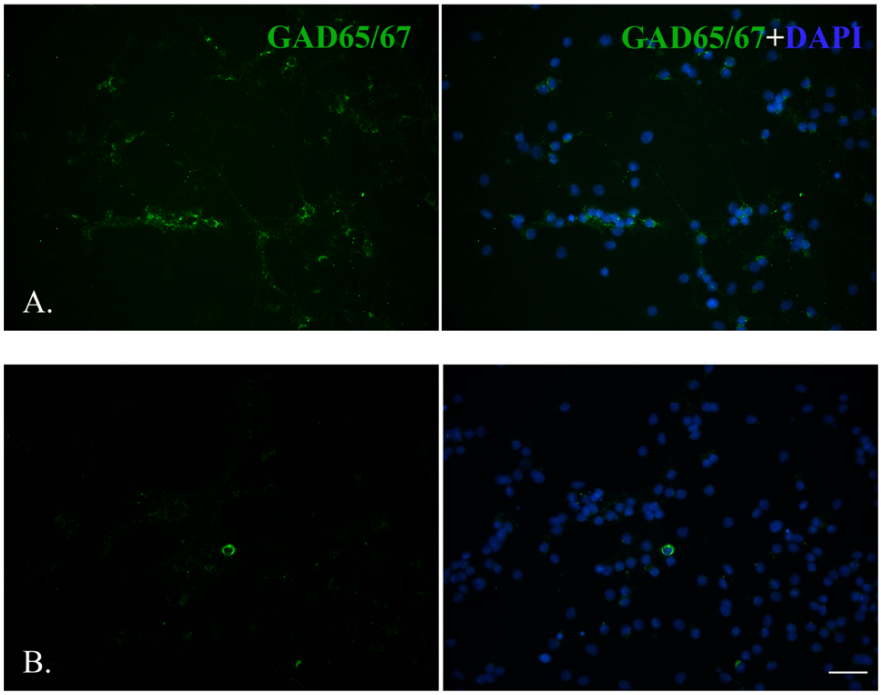


Fig. 5.7. GAD65/67 immunoreactivity identifies inhibitory neurons in hippocampal culture.

GAD65/67(green) immunoreactivity identified inhibitory neurons in hippocampal neuronal culture (DIV 14) counterstained with DAPI (on the right column). **A.** Higher distribution of inhibitory neurons in one microscopic field v/s **B.** Fewer GAD65/67 positive neurons demonstrated heterogeneity in the distribution of inhibitory neurons in culture. (20X objective). Scale bar = 25µm.

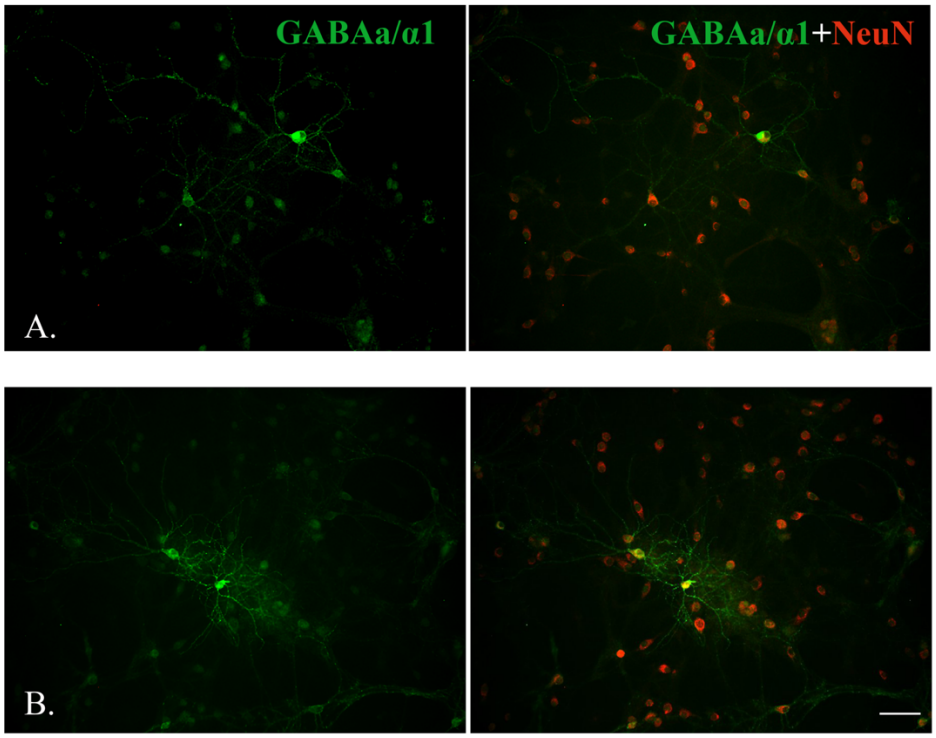


Fig. 5.8. GABAa/α1 immunoreactivity detects inhibitory neurons and synapses in culture.

GABAa/α1 (green) immunoreactivity in specific hippocampal neurons and their processes (DIV 14) counterstained with NeuN (red, right column) to recognize all neurons in the field. To understand heterogeneity in the distribution of neurons expressing GABAa/α1, two microscopic fields are shown here (**A** and **B)** (20X objective). Scale bar = 25µm.

5.4.2.4 Endogenous expression of IL-1 signaling components in primary hippocampal neurons

IL-1β functions through its functional receptor, IL-1R1. IL-1R1 is constitutively expressed primarily in the postsynaptic regions of cultured rat hippocampal neurons, alongside the expression of the receptor accessory protein, IL-1RacP, and the signaling adapter protein MyD88 (Gardoni et al. 2011). IL-1R1(Fig. 5.9), IL-1RacP (Fig. 5.10), and MyD88 (Fig.5.11) immunoreactivity were seen in primary mouse hippocampal neurons in the current study was consistent with previous findings (Gardoni et al. 2011).

P2X7R immunoreactivity was also observed in cultured hippocampal neurons (Fig. 5.12). Sub-cellular localization of IL-1R1 appeared to be in the cell membrane. P2X7R immunoreactivity was concentrated in the cell membrane as well as in neuronal processes.

In vivo, physiological IL-1β is present in the hippocampus of mice brain as seen through ELISA studies (Fig. 4.4). However, IHC studies on the saline-treated mice brain section were not capable of detecting endogenous expression in hippocampus (Fig.3.2, SBE treated brain sections). Unlike absence of immunoreactivity in tissues, ICC staining on hippocampal cells showed endogenous expression of IL-1β in the certain cell population of hippocampal neurons in culture (Fig. 3.3). The IL-1β immunoreactivity was detected in both cell bodies and neuronal processes. Together, these results showed hippocampal neurons in culture endogenously express IL-1β and its signaling components and justified utilization of cultured hippocampal neurons for further characterization of neuromodulatory function of IL-1β (which were done in chapter 3 and 4).

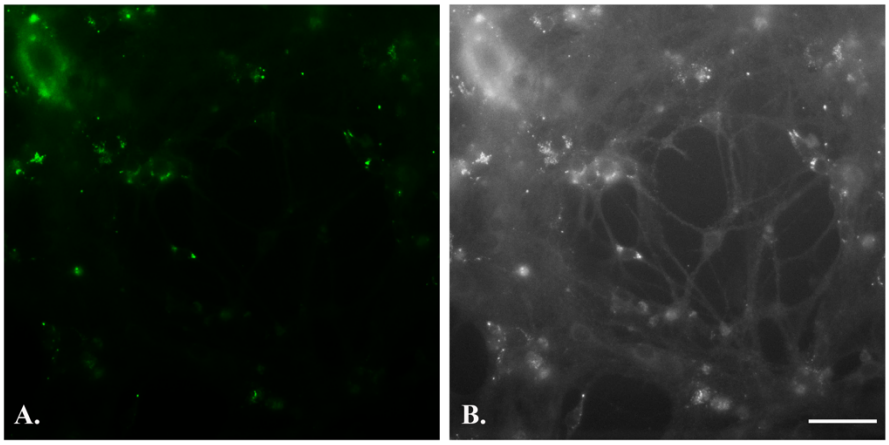


Fig.5.9. Localization of IL-1R1 immunoreactivity in cultured hippocampal neurons.

Photomicrograph of hippocampal neurons (DIV 14) show **A.** IL-1R1 immunoreactivity in green and **B.** gray scale images with IL-1R1 immunoreactivity (40X objective). Scale bar = 25 µm.

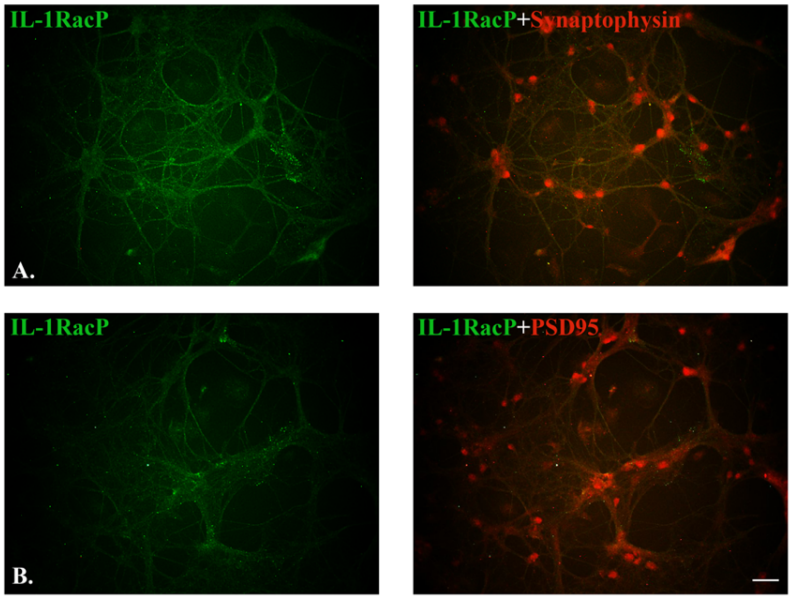


Fig.5.10. Localization of IL-1RacP immunoreactivity in cultured hippocampal neurons.

Photomicrograph of hippocampal neurons (DIV 14) show accessory protein of IL-1 receptor, IL-1RacP immunoreactivity (green) counterstained with **A.** presynaptic marker, Synaptophysin (red) and **B.** postsynaptic marker PSD95 (red) (20X objective). Scale bar = 25 µm.

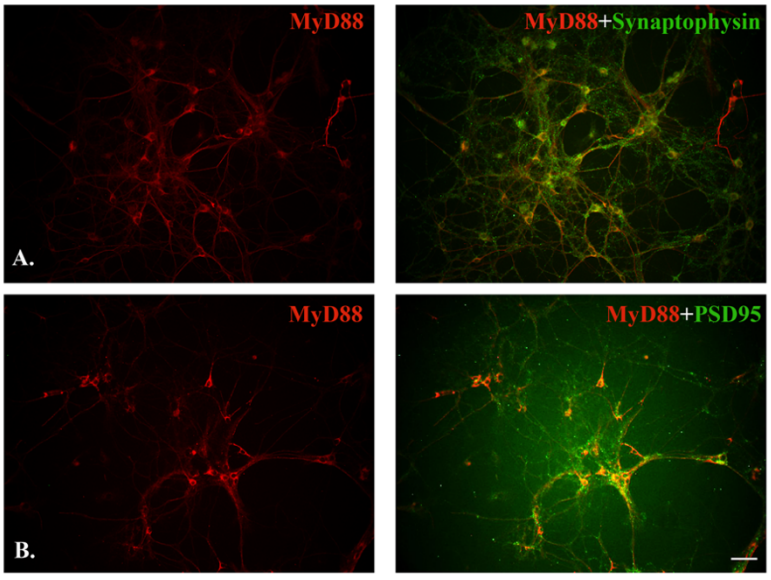


Fig.5.11. Localization of MyD88 immunoreactivity in cultured hippocampal neurons.

Photomicrograph of pure hippocampal neurons (DIV 14) show adaptor protein of IL-1 receptor, MyD88 immunoreactivity (red) counterstained with **A.** presynaptic marker, Synaptophysin (green) and **B.** postsynaptic marker, PSD95 (green) (20X objective). Scale bar = 25 µm.

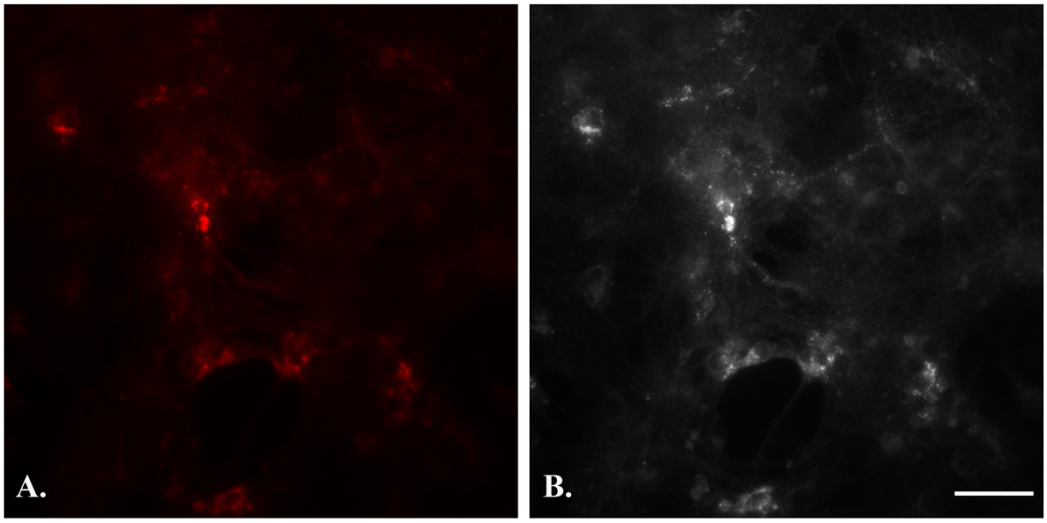


Fig.5.12. Localization of P2X7R immunoreactivity in cultured hippocampal neurons.

Photomicrograph of hippocampal neurons (DIV 14) show **A.** P2X7R immunoreactivity (red) and **B.** gray scale images with P2X7R immunoreactivity (40X objective). Scale bar = 25 µm.

5.5 Concluding remarks

This culture was prepared with a modest level of difficulty and care. Once the protocol was standardized, it was used for a variety of studies, discussed in chapter 3 and 4. Alongside offering a great insight into neuronal physiology and function, this model established itself to be an effective model for this dissertation research.

Chapter 6

Main findings, conclusions and future direction

6.1 Main findings

- IL-1β has role in maintenance of brain homeostasis:
  - Genetic deletion of IL-1R1 in mice lowers seizure threshold – studies from (Claycomb 2011) revisited.
  - Role of P2X7R in modulation of excitatory-inhibitory balance favoring excitation in hippocampal neurons – possibly by blocking IL-1β release.
  - Role of P2X7R in maintaining seizure threshold in mice – possibly blocking IL-1β release.
- There is a presence of endogenous IL-1β protein in neurons of the hippocampus:
  - Endogenous IL-1β expression is present in pyramidal neurons of CA3 and CA1 subregion of the hippocampus.
  - Within hippocampal neurons, IL-1β is present in cell bodies and neuronal processes.
- Constitutive Interleukin-1β release from hippocampal neurons seems P2X7-dependent.
- Intensive neuronal excitation may serve as endogenous stimuli for IL-1β mRNA induction following convulsive stimuli.
- Endogenous IL-1β has potential link to COX-2 expression and functions in modulation of excitatory-inhibitory balance.

6.2 Summary and conclusions : Limitations of current study

6.2.1 IL-1β has role in maintenance of brain homeostasis.

Previous findings from our laboratory research demonstrated how genetic deletion of the IL-1β ligand or its receptor, IL-1R1 lowered the seizure threshold indicating a regulatory nature of IL-1β in maintenance of excitation/inhibition balance. This evidence formed the fundamental basis of this dissertation research. Therefore, as mentioned in chapter 2, prior to characterizing some of key aspects of IL-1β's role in brain homeostasis or delving deeper in endogenous IL-1β biology, this concept was revisited. Convulsant property of PTZ was used to model shift or change E/I balance. PTZ was utilized as it generates intensive excitatory neuronal activity without excitotoxic insults in mice brain (Claycomb, Hewett, and Hewett 2011). Studies herein, showed role of endogenous IL-1β in maintenance of seizure threshold via two independent ways. As it was hypothesized that physiological IL-1β may mediate E/I balance, genetic deletion of IL-1R1 in mice and treating mice with P2X7R antagonist (possible via blocking IL-1β release) demonstrated similar results, proving the hypothesis.

6.2.1.1 Genetic deletion of IL-1R1 in mice lowers seizure threshold – studies from (Claycomb 2011) revisited

One primary concern shown by previous reviewers was developmental defects in these knock out lines generating alterations in their seizure threshold. This may contribute to their seizure phenotype. Deficiency or alterations in phenotypes in mutant mice line is common. Genetic knock-out mice lines may generate alternative phenotypes perhaps due to compensatory changes (Barbaric, Miller, and Dear 2007). However, similar seizure phenotype was observed in IL-1β KO and IL-1R1 KO mice lines (Claycomb, Hewett, and Hewett 2012). Firstly, researchers

developing these lines did not characterize any unusual phenotype in their findings (Zheng et al. 1995; Maliszewski et al. 1997). Secondly, similar phenotypic alterations in two distinct mutant mouse line seem highly unusual. Yet, these proteins belong to the same cytokine signaling pathway and therefore, these lines may generate the similar altered seizure phenotype. Mice lines deficient either the ligand or its receptor, and IL-1 signaling has been implicated in neurogenesis and neuronal development (discussed in chapter 1). Alteration in seizure phenotype may happen due to altered neuronal development and this can pose concern.

6.2.1.2 Role of P2X7R in modulation of excitatory-inhibitory balance – possibly by blocking IL-1β release

Role of P2X7R dependent release of IL-1β in neurons and how it may affect E/I balance was studied and discussed in chapter 3. Utilization of pharmacological inhibitor of P2X7R opened an interesting avenue for studying endogenous neuronal localization of IL-1β and some specific aspects of IL-1β release mechanism which was discussed in section 3.5 of chapter 3.

Inhibiting P2X7R activation displayed accumulation of IL-1β both *in vivo* and *in vitro* (Chapter 3). This indicated role of P2X7R in endogenous IL-1β release from neurons. P2X7R antagonism study demonstrated altered neuronal excitation *in vitro* and lowered seizure threshold *in vivo*. Of interest to this section, following P2X7R inhibition, PTZ induced seizures were more severe in mice (Chapter 3). Alongside, connected to the topic of IL-1β's role in maintenance of seizure threshold, pharmacological inhibition of P2X7R activation yielded similar results to PTZ induced seizure studies with genetic deletion of IL-1β ligand (Claycomb, Hewett, and Hewett 2012) or its receptor [(Claycomb, Hewett, and Hewett 2012) and chapter 2].

Lowered seizure threshold by pharmacological inhibition of P2X7R possibly happens via blocking IL-1β release, therefore, this study may provide additional evidence for role of

endogenous IL-1β in the maintenance of E/I balance and may bypass some concerns associated with utilization of mutant mice lines. As the effect of this drug was acute, it can be argued that developmental effects did not likely account for the seizure phenotypes seen with IL-1β ligand or the receptor knock out mice. In this aspect, the genetic and pharmacological approach together provided compelling evidence to support the contention of IL-1 signaling in the maintenance of seizure threshold.

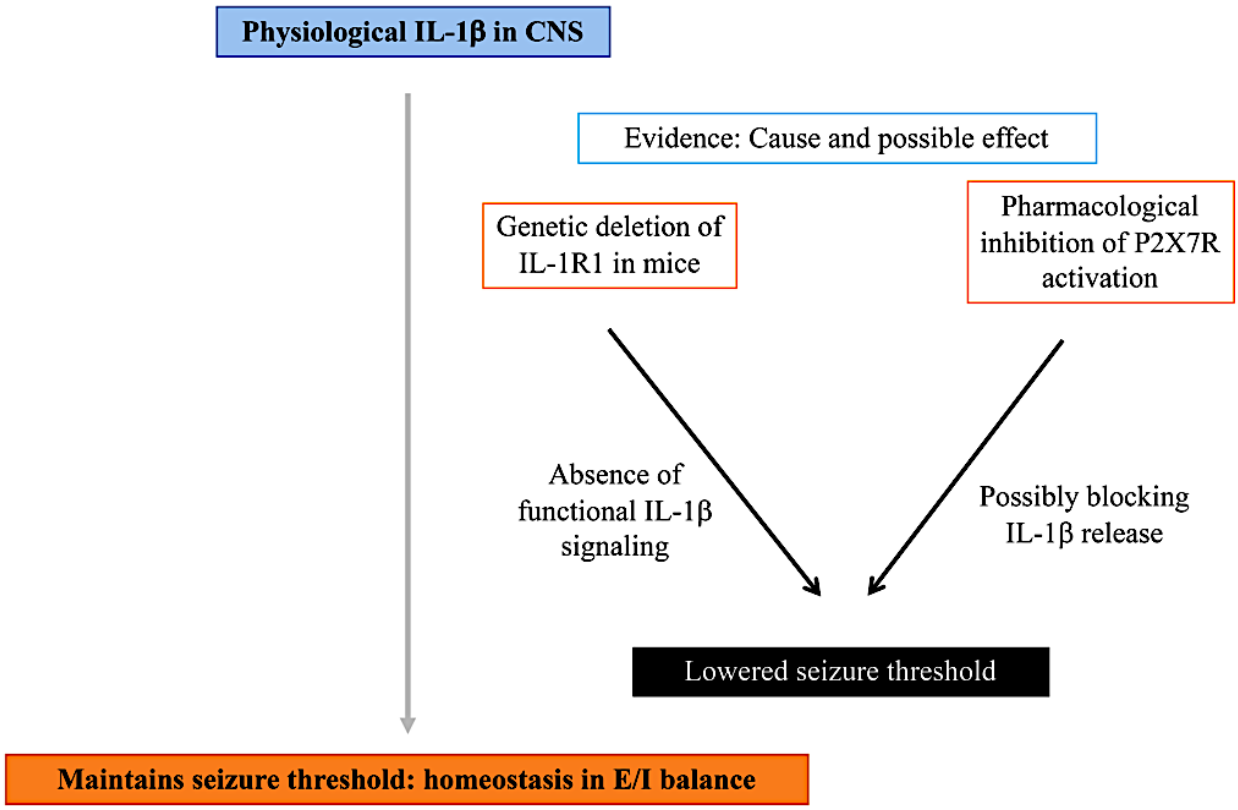


Fig. 6.1. Physiological IL-1β in CNS maintains seizure threshold.

6.2.2 Endogenous IL-1β expression in neurons of the hippocampus.

6.2.2.1 Cellular localization: CA3/CA1 pyramidal neurons

Localization of IL-1β in CNS have been classified in literature based on different sub-regions of the brain where it is either expressed [endogenous expression in the hypothalamus (Breder, Dinarello, and Saper 1988) and hippocampus (Lechan et al. 1990; Deak, Bellamy, and D'Agostino 2003) or whether its condition of release is physiological (primarily neuronal) (Watt and Hobbs 2000) or pathophysiological (primarily microglial) (Liu and Quan 2018). The localization or source of endogenous IL-1β have been discussed in chapter 1 and section 3.5.1 of chapter 3. Few explanations were also discussed to justify the incongruency between findings from different researchers on the cellular sources of IL-1β in mice hippocampus.

In CNS, microglia are the primary source of IL-1β in neuroinflammatory and neurodegenerative conditions. Several reviews over the years have tied information from literatures and helped in better understanding of cellular sources of IL-1β in pathophysiological conditions (Allan, Tyrrell, and Rothwell 2005; Dinarello 2009; Hewett, Jackman, and Claycomb 2012; Liu and Quan 2018). During neuroinflammation, initial source of IL-1β is microglial, however, with continuation of inflammatory and subsequent reparative processes, IL-1β is also sourced from reactive astrocytes, neurons and even from endothelial cells (Boutin et al. 2003).

On the other hand, information on the physiological source of IL-1β seem to be compartmentalized as the researchers till now have only described their region of interest in CNS. Two areas of brain has been widely studied in respect to endogenous role of IL-1β, hypothalamus, particularly in hormone regulation and central stress axis (Watt and Hobbs 2000; Goshen and Yirmiya 2009) and in hippocampus primarily with LTP and learning and memory processes (Schneider et al. 1998; Curran, Murray, and O'Connor 2003; Ikegaya et al. 2003;

Viviani et al. 2014). Therefore, most information available on cellular localization of endogenous IL-1β have been derived from these two regions of the brain. Finally, most studies done to explore role of IL-1β physiology utilized an exogenous trigger (like LPS) or exogenous IL-1β (Ross et al. 2003; Goshen et al. 2007). Although these studies claimed exogenous IL-1β level were at physiological range, it is challenging to conclude if it actually mimicked the physiological condition. Along the same line of thought, most physiological roles of IL-1β was initially identified alongside specific disease conditions such as memory impairment due to fever, sickness behavior or neurodegenerative conditions (Spulber and Schultzberg 2010), for which pinpointing the cellular source of IL-1β was made further difficult.

Studies done in rat brain to measure IL-1β level in different regions indicated its low physiological level in hypothalamus, hippocampus and in posterior cortex of the brain (Deak, Bellamy, and D'Agostino 2003; Porterfield et al. 2012). Study herein similarly showed very low (picogram level) IL-1β in the hippocampus of mice brain via ELISA study (Fig.4.3, Chapter 4). As IL-1β level was low, it remained undetected with IHC study, hence the localization still remained unclear. Only when P2X7R was inhibited, endogenous IL-1β accumulated in pyramidal neurons of CA3 and CA1 sub-region of hippocampus (Fig.3.1).

Firstly, as discussed in section 3.5.1 of Chapter 3, variation of expression between species can owe to differential expression found between physiological IL-1β expression in rats v/s current result obtained here in mice. However, it still does not explain for the fact as to why there are different expression pattern seen by different researchers in the same species (rat) (Lechan et al. 1990; Kaneko et al. 2006; Viviani et al. 2014). Secondly, observation in the current study is based on accumulation caused by P2X7R inhibition. Studies herein showed similar expression pattern in CA3 and CA1 region as seen in Viviani et al, however, no IL-1β expression was seen

in DG. Although P2X7R was indicated to be involved in endogenous IL-1β release from neurons, further investigation is required to understand if any other parallel release mechanism exists which may explain absence of IL-1β expression in DG.

Hippocampal neurons in culture also showed IL-1β immunoreactivity, however, unlike in brain section, where IHC was unable to detect any immunoreactivity, certain populations of hippocampal neurons in culture showed endogenous IL-1β immunoreactivity. The hippocampal neuron culture system served as a tool to better understand the subcellular localization of IL-1β within neurons.

6.2.2.2 Sub-cellular localization of IL-1β in neurons of hippocampus

The expression pattern of IL-1β immunoreactivity in pyramidal neurons of CA3 and CA1 neurons was consistent to the IL-1β expression as seen with CA3 neurons in normal hippocampus of rats (Viviani et al. 2014) and IL-1β immunoreactivity seen in hypothalamic neurons (Watt and Hobbs 2000). The CA3/CA1 pyramidal neurons showed similar perinuclear punctate profile similar to neurons of hypothalamus (Watt and Hobbs 2000). However, the current study was lacking any IL-1β presence in neuronal processes *in vivo* as observed in neuronal fibers (Watt and Hobbs 2000). A caveat of the current study lies in not conducting colocalization studies with marker for neuronal processes. Colocalization study with MAP-2 or β-III-tubulin in brain section treated with JNJ may provide answer, whether IL-1β is localized in neuronal processes.

Using higher resolution microscopy, sub-cellular localization of hippocampal neurons was investigated.

6.2.2.3 Sub-cellular localization of IL-1β in cultured hippocampal neurons

Epifluorescence microscopy study on cultured hippocampal neurons showed IL-1β immunoreactivity in both cell bodies (similar to *in vivo* observation) and in neuronal processes in similar punctate profiles as discussed above (Watt and Hobbs 2000; Lechan et al. 1990). Sub-cellular localization of IL-1β was further examined in primary hippocampal neuron culture using high resolution microscopy. Higher-resolution microscopy study showed IL-1β having a punctate profile in the cell body and neuronal processes, alongside some diffused immunoreactivity. Endogenous cytosolic expression of IL-1β in cultured neurons was consistent with sub-cellular localization pattern of IL-1β found in cultured murine macrophage, showing similar perinuclear cytosolic expression (Brough and Rothwell 2007). Although, different cell types utilize variant form of IL-1β production and release mechanism (chapter 1), it was found cytosolic IL-1β localization pattern across cell types remained consistent.

Few interpretations can be made based upon the sub-cellular localization of IL-1β in neurons. Vesicle like structures seen herein may be IL-1β packaged in microvesicles, required for IL-1β release (Rubartelli et al. 1990; Monteleone et al. 2018). Further experimentation will be required to understand the mechanism clearly.

It is also important to investigate whether endogenous IL-1β release occurs synaptically or extra-synaptically. A recent study using a novel transgenic approach to map IL-1R1 protein expression in the hippocampal formation found prominent IL-1R1 protein in the cell soma and molecular layer of DG granule cells (Liu et al. 2015). The latter is consistent with another study showing post-synaptic localization of IL-1R1 in hippocampal neurons (Gardoni et al. 2011). Expression was also detected in the mossy fiber tract of the DG neurons, suggesting a possible presynaptic release of IL-1β. Vesicles observed in the neuronal processes might be way to transport IL-1β to

the synaptic locations or indication of pre-synaptic sites, however, further investigations will be required prior to remarking. As IL-1β release requires P2X7R activation which in turn opens pore for IL-1β release, sub-cellular localization of P2X7R may also be important in understanding cellular sites for IL-1β release.

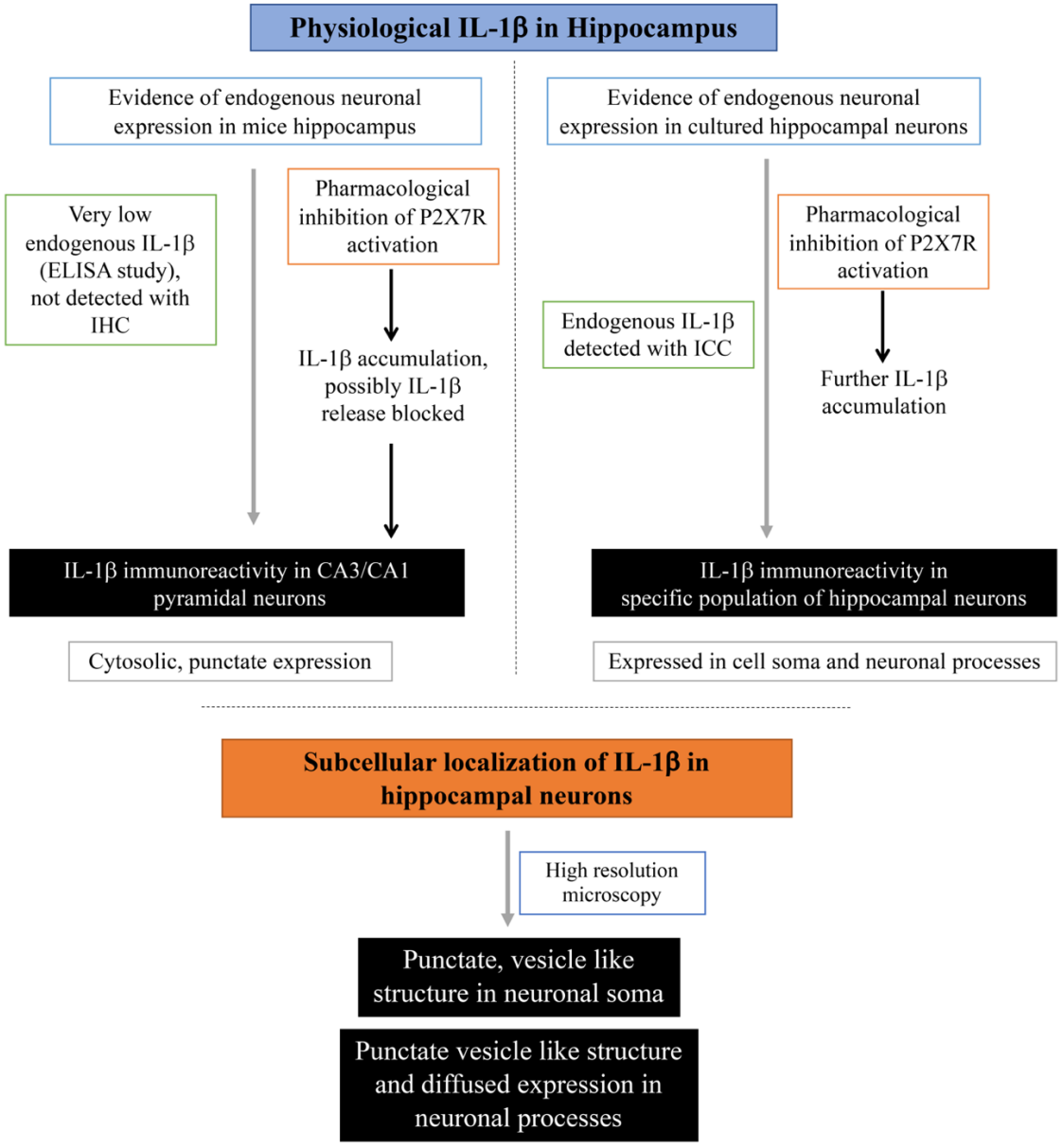


Fig.6.2. Physiological IL-1β expression in mice hippocampus is present in CA3/CA1 pyramidal neurons and its subcellular localization shows punctate vesicle like structure in cell soma and neuronal processes.

6.2.3 Endogenous IL-1β release from the neuron may be similar to canonical release pathway – P2X7R dependent

Studies herein have implied inhibiting P2X7R activation possibly blocked IL-1β release from hippocampal neurons. Both *in vivo* and *in vitro*, P2X7R antagonism caused accumulation of endogenous IL-1β in neurons. P2X7R antagonism also shifted the E/I balance favoring excitation in hippocampal neurons and lowered seizure threshold in mice. However, it was acknowledged in section 3.5.3 of chapter 3, that the effect of the P2X7R antagonist on IL-1β release and E/I balance are correlative yet results herein did not directly addressed cause and effect. P2X7R is an ATP receptor, which itself functions as a neurotransmitter in CNS (Burnstock 2016), thereby P2X7R can affect E/I balance independent and/or parallel to neuromodulation by IL-1β. In the following sections, effect of both possibilities will be discussed to form alternative theories and understand endogenous release of IL-1β from hippocampal neurons.

6.2.3.1 P2X7R may be required for physiological IL-1β release from neuron

As discussed in chapter 1, P2X7R in CNS has following functions, firstly it is a low-affinity ATP receptor, which when activated by ATP, opens a non-selective pore for extracellular ATP release, IL-1β release and acts as stimuli to activate inflammasome (Sanz and Virgilio 2000; 2000; Sperlagh et al. 2006; Giuliani et al. 2017). Studies herein have shown pharmacologically inhibiting P2X7R accumulated endogenous IL-1β in hippocampal neurons possibly via blocking its release. Furthermore, in inflammatory cells, where IL-1β release have been thoroughly studied, even though mechanism of IL-1β processing and packaging for release varied [microvesicle shedding (Monteleone et al. 2018), secretory lysosome release (Rubartelli et al.

1990; Andrei et al. 1999) or exosome secretion (Qu et al. 2007)], every release mechanism from these cells have been dependent on P2X7R activation (Ferrari et al. 2006; Giuliani et al. 2017). Herein, parallels are drawn from other studies, which may indicate presence of similar mechanism by which P2X7R dependent IL-1β release may occur in neurons, even in absence of inflammatory stimuli.

IL-1β release occurs in response to different endogenous or exogenous stimuli and some of its mechanism are varied based on cell types (Piccioli and Rubartelli 2013). IL-1β release occurs after it is packaged in some form of vesicle (microvesicles or secretory lysosomes or exosome). In CNS, IL-1β is released from microglia by microvesicle shedding (Bianco et al. 2005). In hypothalamic neurons, osmotic challenge led to complete loss of similar IL-1β containing vesicle like structure (Watt and Hobbs 2000). Although studies herein have shown punctate IL-1β immunoreactivity in cell soma and neuronal processes (similar vesicle like structure), further investigation will be required to understand , 1) if IL-1β in hippocampal neurons are indeed packaged in these vesicles for secretion, and 2) these vesicle-like structures are released from neurons.

Secondly, sub-cellular localization of IL-1β release will depend on P2X7R localization in neurons. P2X7R has pre-, post, and extra-synaptic localization in neurons (Miras-Portugal et al. 2017), each with specific functions. P2X7R activation also open different sized pores based on neuronal conductance (Alves et al. 2014). Further experimentation will be required to understand which localization of activated P2X7Rs required for neuronal IL-1β release.

6.2.3.1 Possible source of ATP and Caspase-1 in hippocampal neurons

Studies herein implied P2X7R dependent IL-1β release in neurons. P2X7R is an ATP receptor. The logical question that followed next was to identify the possible source(s) of ATP to activate P2X7R for IL-1β release. Furthermore, IL-1β release coincidence with IL-1β processing (Rubartelli et al. 1990; Andrei et al. 1999; Piccioli and Rubartelli 2013). Canonical inflammatory processing of IL-1β requires functional Caspase-1, which cleaves pro-IL-1β to its active form (Verhoef et al. 2003; Burns, Martinon, and Tschopp 2003; Brough and Rothwell 2007; Denes, Lopez-Castejon, and Brough 2012). In the following section, likely source of ATP and possible involvement of Caspase-1 will be discussed.

Source of ATP for P2X7R activation: It is pertinent to identify the physiological source of ATP, which is required for P2X7R activation required for IL-1β release. P2X7R is a low-affinity receptor for ATP, therefore, a sufficient concentration of ATP is required for P2X7R activation. ATP is a readily available molecule in the nervous system milieu, being produced and released by both neurons and glial cell types (Sperlagh et al. 2006; Sperlágh and Illes 2014). ATP acts either as a neurotransmitter or as a neuromodulator to affect neurotransmitter functions. It is taken up and released in synaptic vesicles in nerve terminals. ATP is released during neuronal activity and can activate P2X7R receptors in narrow synaptic clefts (Sperlagh et al. 2006).

Owing to neurotransmitter functions, synaptic release of ATP is an obvious possibility (Pankratov et al. 2006). Astrocytes are also known to release ATP in a glutamate-dependent manner (Koizumi et al. 2003; Zhang et al. 2003) and microglia can release ATP via exocytosis under certain conditions (Imura et al. 2013). Combinations of these cells can contribute to extracellular ATP (Lalo et al. 2016).

Further investigation will be required to better understand the source of ATP *in vivo*, required for P2X7R activation for IL-1β release from neurons. P2X7R dependent IL-1β release from cultured hippocampus, however, might provide some hint. They are near pure cultures devoid of glial cells. ATP required for P2X7R activation in culture may be sourced from the hippocampal neurons itself, however, further experimentation is needed to confirm this possible mechanism .

Requirement of Caspase-1 - possible involvement of inflammasome: Preliminary study herein have shown that pharmacologically blocking Caspase-1 in cultured hippocampal neurons elevated pro-IL-1β level in cell lysate. Absence of active IL-1β in cell lysate could have occurred due to either of the two reasons. Firstly, in hippocampal neurons, processing of IL-1β may occurs parallel to release, thereby processed IL-1β may only be available post release. Secondly, active IL-1β bound for release was packaged in microvesicles and got separated in the layer containing vesicles during centrifugation, therefore absent in lysate supernatant. My study indicated the possibility of caspase-1 functionality (Fig.7.8, Appendix). This would also suggest that processing as well as release of IL-1β occurs in a P2X7R-dependent manner in neuron as reported in inflammatory cells (Piccini et al., 2008).

Caspase-1 is processed from pro-caspase-1 after it is integrated into the inflammasome (van de Veerdonk et al. 2011). Therefore, the involvement of Caspase-1 also opens the possibility of inflammasome mediated IL-1β processing. Different elements of the inflammasome are expressed in CNS and are implicated in CNS physiology and pathophysiology (Xu et al. 2018; de Rivero Vaccari, Dietrich, and Keane 2014; Walsh, Muruve, and Power 2014; Kaushal et al. 2015; Heneka, McManus, and Latz 2018). Alternatively, inflammasome complex formation might not occur in endogenous IL-1β release from neurons. An alternative model present in monocytes shows the presence of ASC and NLRP, which forms a platform for constitutive

Caspase-1 activation (Netea et al. 2010). A similar protein platform may be present in neurons instead of the inflammation mediated multimeric protein complex (inflammasome) formation for constitutive Caspase-1 activation which may be required for constitutive release of endogenous IL-1β. How processing occurs in neurons under physiological conditions remains to be determined, although based on studies herein, it is presumed to occur in a P2X7-induced caspase-1-dependent manner.

6.2.4 Endogenous stimuli for IL-1β production

IL-1β mRNA induction in immune cells require exogenous (bacterial toxin, LPS) or endogenous (inflammatory mediators, cytokines) stimuli for transcription and subsequent translation of IL-1β (Fenton 1992; 1988; Pelegrin, Barroso-Gutierrez, and Surprenant 2008). Activation of TLR is required of IL-1β induction in CNS pathophysiology. Constitutive production of IL-1β transcript have only been reported in human large granular lymphocytes and in male gonads (Galli et al. 1990; Rozwadowska et al. 2007). Constitutive generation of constitutive IL-1β transcript in neurons is unknown. This would indicate transcription and translation of IL-1β in neuron may require a stimulus. In the absence of inflammatory stimuli, the question remains as to what triggers physiological IL-1β production in neurons.

Recent studies have found several sterile stimuli can initiate a response to release IL-1β (Patel et al. 2016). One such candidate identified for priming inflammasome is Reactive Oxygen Species (ROS). Metabolism of mitochondrial energy yields metabolites including ROS in all living cells including cells of CNS. ROS have both detrimental and beneficial roles in neuronal physiology, based on its level and stimuli for production (Cid-Castro, Hernández-Espinosa, and Morán 2018) and implicated in several neurological diseases (Popa-Wagner et al. 2013; Angelova and

Abramov 2018). However, whether ROS is a possible candidate to initiate endogenous IL-1β in CNS physiology is not known. The brain is a high energy-consuming organ which generates a substantial amount of ROS, however, it also has a prominent antioxidant system to limit oxidative stress (Salim 2017). In a homeostatic system of functional oxidative phase, a limited low amount of mitochondrial metabolite may function as a stimulus to produce endogenous IL-1β. Information on this endogenous stimulus that may initiate physiological IL-1β in neurons is unknown.

Conversely, studies herein have shown intensive neuronal excitation can act as an endogenous trigger to rapidly induce IL-1β mRNA in mice hippocampus. Section 4.5.1 discussed in detail, instances where neuronal activity have induced IL-1β. Therefore, thorough research needs to be done to understand the possibility of ROS or basal neuronal activity as a potential trigger in endogenous IL-1β production in the neuron.

6.2.5 COX-2 as a downstream signaling candidate of IL-1β

COX-2 mRNA levels showed dissimilar outcomes when IL-1β signaling was disrupted. Studies herein demonstrated P2X7R inhibition increased COX-2 mRNA levels in hippocampal neurons (Chapter 3). On other hand, IL-1R1 KO mice demonstrated no difference in constitutive hippocampal COX-2 mRNA compared to WT (Chapter 4). The first instance can be explained by the possible sequence of event, where P2X7R inhibition possibly blocked IL-1β release which in turn shifted the E/I balance favoring excitation (demonstrated through elevated c-Fos level). This in turn induced COX-2 mRNA. Spontaneous glutaminergic activity have shown to induce COX-2 mRNA, which is consistent with this finding (Yamagata et al. 1993; Stark and Bazan 2011; Hewett et al. 2016).

This differs from how endogenous IL-1β may modulate COX-2 mRNA in IL-1R1 mutant mice. As demonstrated in chapter 4 and discussed in section 4.5.2, absence of functional IL-1β signaling in mice did not affect the basal COX-2 mRNA levels. However, it moderated basal COX-2 expression in hippocampus possibly via maintaining post-transcriptional COX-2 mRNA stability (Tamura et al. 2002).

Secondly, activity dependent COX-2 expression was also moderated by endogenous IL-1β signaling. COX-2 expression was induced with PTZ induced convulsive seizure. This is consistent with previous findings (Claycomb, Hewett, and Hewett 2011; Gong and Hewett 2018). Studies herein showed activity dependent COX-2 induction even in the IL-1R1 KO mice with PTZ treatment, however in lesser extent. This indicated IL-1β - COX-2 link.

Endogenous IL-1β regulates activity dependent COX-2 possibly via regulation of NMDA function. IL-1β enhanced NMDA function whereas inhibiting IL-1β abolished this effect (B Viviani et al. 2003). Also, IL-R1 is coupled with NR2B receptor subunit in post synaptic density of cultured hippocampal neurons (Gardoni et al. 2011). Absence of functional IL-1R1 in KO mice therefore, may compromise NMDA receptor function, creating possibility of attenuated NMDAR function in the mutant mice. As induction of COX-2 is shown to be dependent on glutaminergic signaling, this in turn, may affect COX-2 expression. NMDAR also mediates COX-2 dependent PG synthesis (Stark and Bazan 2011). This can explain lower PG levels in hippocampus of mutant mice following convulsion.

Identifying endogenous IL-1β dependent NMDAR function will require further experimentation. Identifying sub-cellular localization of IL-1β will be required to understand the above-mentioned possibilities. Excitatory activity dependent COX-2 is expressed in the dendritic spine of CA3 pyramidal neurons (Kaufmann et al. 1996). IL-1R1 also demonstrates post-synaptic localization

(Gardoni et al. 2011; Liu et al. 2019). This may indicate a possibility that neuronal IL-1β is released from pre-synaptic location. Studies herein have shown IL-1β immunoreactivity in neuronal processes. Further experimentation will be required to understand if these immunoreactivity colocalizes with the presynaptic sites, to identify possible release sites of IL-1β in neurons.

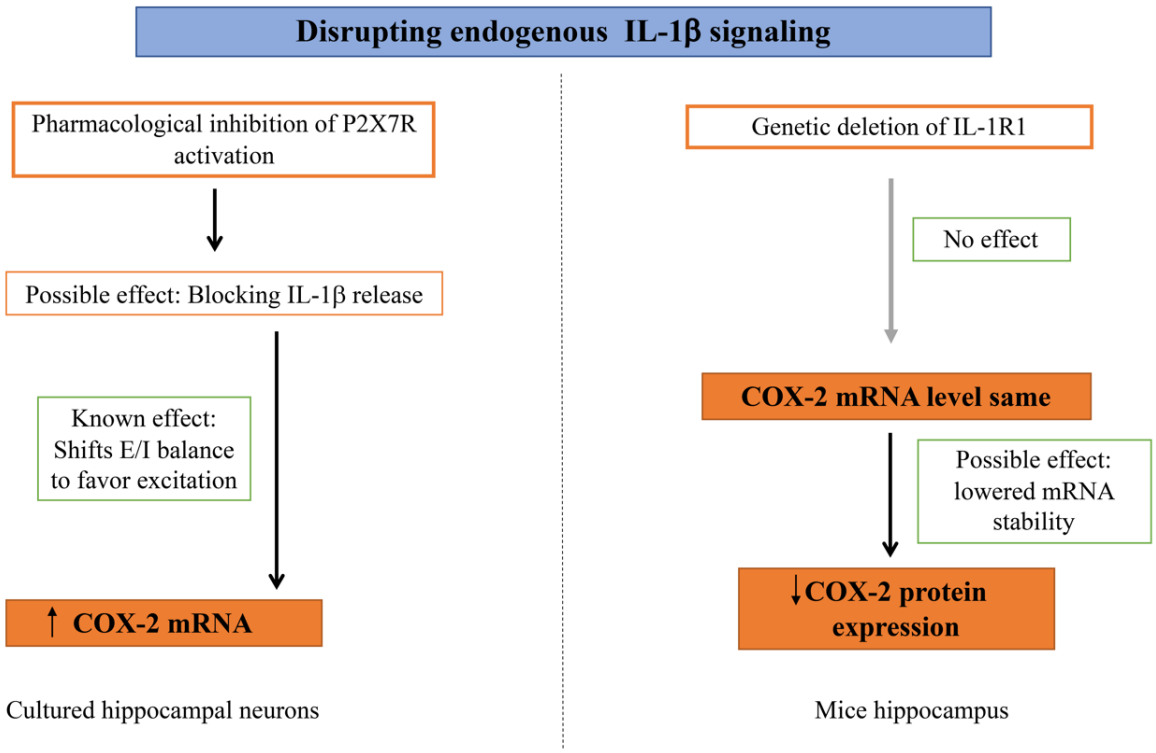


Fig. 6.3. Dual outcome of COX-2 mRNA induction by disruption of endogenous IL-1β signaling.

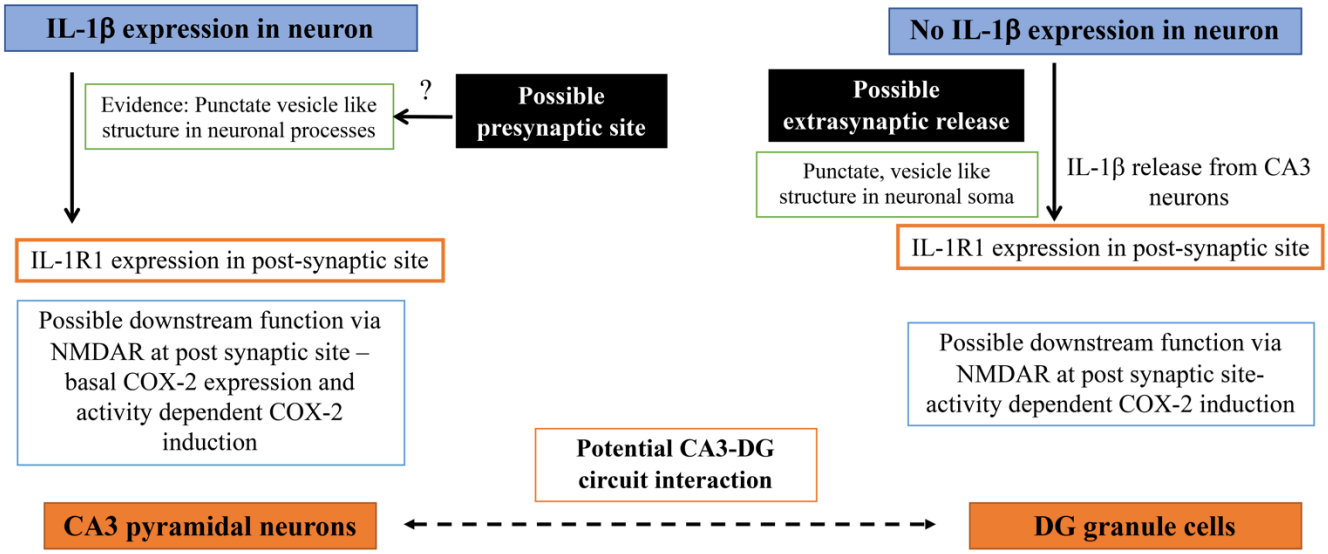


Fig. 6.4. Potential location for IL-1β-COX-2 functioning in mice hippocampus.

6.3 Future directions

6.3.1 Identifying similarities and uniqueness of the physiological release of IL-1β to the canonical inflammatory release mechanism.

This dissertation research identified physiological presence of IL-1β in neurons of the hippocampus. Simultaneously, JNJ studies done herein have shown, physiological IL-1β release may be P2X7R activation dependent.

Inflammatory IL-1β release requires two distinct signals for its release, firstly, it induces IL-1β mRNA and pro-IL-1β protein, and secondly, initates formation of the inflammasome to activate Caspase-1 when P2X7R is activated by extracellular K^+ efflux and ATP release. How these signals may originate without inflammation to release IL-1β in CNS physiology was not studied in my dissertation research.

The first logical future step in further characterization of IL-1β release mechanism in neuron would be to identify the cellular source of ATP and possible triggers that will release ATP into the extracellular milieu. Secondly, study herein also indicated physiological IL-1β release may require Caspase-1. Further investigation will be required to identify, how Caspase-1 may play a role in IL-1β release in neurons, and finally, whether or not inflammasome plays a role in activating Caspase-1.

6.3.2 Identifying the DG-CA3 circuitry as IL-1β communication center in the hippocampus.

Studies in section 7.6 (Appendix) explained the initiative and progression of research involving use of two mouse lines. The first of these targeted Cre-DNA recombinase to DG granule cells using the proopiomelanocortin (Pomc) gene promoter (Pomc-Cre; (McHugh et al. 2007)). This promoter is transcriptionally activated during the late postnatal period of development, lessening the possibility of a developmental effect of the transgenic manipulation. The second mouse line

harbored a functional IL-1RI transgene that is transcriptionally activated by Cre in the background of global IL-1RI gene deficiency (Liu et al. 2015). The transgenic construct was inserted in the endogenous IL-1RI gene, thus inactivating it. The research plan was to activate IL-1R1 signaling in DG of the hippocampus and verify the seizure phenotype in lights of IL-1 signaling recovery in DG.

However, as explained in the section 7.6 (appendix), there were several unprecedented problems in the breeding and maintenance of the transgenic line which attenuated the progression of this research.

Genetic deletion of IL-1β signaling from the DG of adult mice would have been a logical approach to test my hypothesis of possible localization of IL-1β functioning in hippocampus. However, during the origin of this project, a mouse line with loxP-flanked IL-1RI which permits spatial deletion of IL-1 signaling was yet to be generated. Recently, there is a functional IL-1R1 conditional knock out mouse line (Robson et al. 2016) which can be utilized to knock out functional IL-1 signaling in DG of the hippocampus and observe the changes in seizure phenotype. Although similar characterization and breeding protocols have to be followed prior to generation of double transgenic knock-out, the positives of this project will be, i) it will be one less step in breeding procedure (no back cross required), and ii) this is a line generated in C57BL/6J strain, similar to the POMC-Cre mouse line instead of IL-1R1 restore line which was is C57BL/6N strain (Robson et al. 2016; Liu et al. 2015). Although my study was not able to pinpoint any of un-precedented problems in breeding with my study due to crossing between two strains, however, above suggested study can be an alternative to answer our research question.

8.3.4 Possible neuroprotection of IL-1β via regulation of post-ictal suppression.

Studies herein (Chapter 4) found induction of IL-1β and Caspase-1 mRNA following an acute seizure. However, there was no immediate change in IL-1β protein level with neuronal hyperexcitation both *in vivo* and *in vitro*. Studies herein also showed IL-1β modulated elevated COX-2 levels and Prostaglandin E_2 production in mice following convulsive seizure (Chapter 4). Several PGs have shown to have anti-convulsive properties (Kim et al. 2008). It is a plausible extension of this study to identify the changes in other prostaglandins particularly of PGD_2 with PTZ induced acute convulsion and to identify the role of IL-1β in PG production. PGD_2 is shown to be the most prevalent prostaglandin in the brain and have an anticonvulsive property (Förstermann et al. 1982; Akarsu, Mamuk, and Comert 1998). Finding the PGD_2 level alongside already obtained PGE_2 data can lead to the next step in identifying the role of IL-1β signaling in post-ictal suppression.

Acute ictal events are typically terminated spontaneously within seconds to several minutes and are followed by a postictal seizure refractory period that may be linked mechanistically to seizure resolution (Mucha and Pinel 1977). Elevated levels of PGs due to PTZ induced convulsion have shown to contribute to post ictal suppression (Förstermann et al. 1982). IL-1 signaling has also been implicated in post-ictal suppression utilizing a different model of seizure generation (Tao et al. 2015). To test whether IL-1β signaling contributes to this postictal suppression, maximal electroshock convulsion (MES) will be given to IL1RI WT and IL1RI KO littermates which will be followed by a second challenge with a convulsive dose of PTZ at time intervals described (Förstermann et al. 1982) and the seizure phenotype has to be studied [the reason for selecting two different modes of seizure generation is to avoid any build-up of the similar stimulus (the second dose of PTZ immediately after another amplifies seizure responses (unpublished

observation)]. If IL-1 serves a role in the postictal refractory period following a PTZ-induced convulsion as hypothesized, a second seizure response would not occur in IL-1RI WT mice within the postictal time period. In contrast, IL-1RI$^{-/-}$ mice may respond during this period. This outcome seems plausible since IL-1RI$^{-/-}$ mice exhibit a reduced seizure threshold. Alternatively, IL-1β signaling may not have any role in post-ictal suppression or already elevated levels of prostaglandin in IL-1R1 KO mice may keep the refractory time period similar (Results from this dissertation research (Chapter 4) shows elevation in PGE_2 level in WT mice whereas is KO mice already elevated levels do not change with convulsion). In this case, the seizure response following the initial convulsion would be suppressed in both IL-1RI$^{-/-}$ and IL-1RI$^{+/+}$ mice. The outcome would be informative as it is important to understand the mechanisms involved in postictal seizure suppression because it could lead to the development of new antiepileptic therapies.

Chapter 7: Appendix

Table 7.1 Individual *p* values (for multiple comparisons) of each pixel points between treatments for Figure 3.7.

Uncorrected Fisher's LSD	Significant?	Summary	Individual *p*-Value
Row 1			
Control vs. 0.1 μM JNJ	No	ns	0.6194
Control vs. 0.3 μM JNJ	Yes	****	<0.0001
Row 2			
Control vs. 0.1 μM JNJ	No	ns	0.6384
Control vs. 0.3 μM JNJ	Yes	***	0.0002
Row 3			
Control vs. 0.1 μM JNJ	No	ns	0.9697
Control vs. 0.3 μM JNJ	Yes	***	0.0008
Row 4			
Control vs. 0.1 μM JNJ	No	ns	0.8348
Control vs. 0.3 μM JNJ	Yes	**	0.0025
Row 5			
Control vs. 0.1 μM JNJ	No	ns	0.4894
Control vs. 0.3 μM JNJ	Yes	**	0.0018
Row 6			
Control vs. 0.1 μM JNJ	No	ns	0.3749
Control vs. 0.3 μM JNJ	Yes	**	0.0011
Row 7			
Control vs. 0.1 μM JNJ	No	ns	0.2736
Control vs. 0.3 μM JNJ	Yes	**	0.0011
Row 8			
Control vs. 0.1 μM JNJ	No	ns	0.2977
Control vs. 0.3 μM JNJ	Yes	***	0.0009
Row 9			
Control vs. 0.1 μM JNJ	No	ns	0.1888
Control vs. 0.3 μM JNJ	Yes	***	0.0005
Row 10			
Control vs. 0.1 μM JNJ	No	ns	0.207
Control vs. 0.3 μM JNJ	Yes	***	0.0006
Row 11			
Control vs. 0.1 μM JNJ	No	ns	0.2824
Control vs. 0.3 μM JNJ	Yes	***	0.0006
Row 12			
Control vs. 0.1 μM JNJ	No	ns	0.3299
Control vs. 0.3 μM JNJ	Yes	***	0.0005
Row 13			
Control vs. 0.1 μM JNJ	No	ns	0.3238
Control vs. 0.3 μM JNJ	Yes	***	0.0003
Row 14			
Control vs. 0.1 μM JNJ	No	ns	0.429
Control vs. 0.3 μM JNJ	Yes	***	0.0004
Row 15			
Control vs. 0.1 μM JNJ	No	ns	0.4442
Control vs. 0.3 μM JNJ	Yes	***	0.0005
Row 16			
Control vs. 0.1 μM JNJ	No	ns	0.6142
Control vs. 0.3 μM JNJ	Yes	**	0.0025
Row 17			
Control vs. 0.1 μM JNJ	No	ns	0.7065
Control vs. 0.3 μM JNJ	Yes	**	0.0054
Row 18			
Control vs. 0.1 μM JNJ	No	ns	0.7007

Control vs. 0.3 µM JNJ	Yes	**	0.0063
Row 19			
Control vs. 0.1 µM JNJ	No	ns	0.3737
Control vs. 0.3 µM JNJ	Yes	**	0.0068
Row 20			
Control vs. 0.1 µM JNJ	No	ns	0.2919
Control vs. 0.3 µM JNJ	Yes	**	0.0083
Row 21			
Control vs. 0.1 µM JNJ	No	ns	0.3284
Control vs. 0.3 µM JNJ	Yes	**	0.0062
Row 22			
Control vs. 0.1 µM JNJ	No	ns	0.5618
Control vs. 0.3 µM JNJ	Yes	*	0.0188
Row 23			
Control vs. 0.1 µM JNJ	No	ns	0.8157
Control vs. 0.3 µM JNJ	Yes	*	0.0357
Row 24			
Control vs. 0.1 µM JNJ	No	ns	0.6254
Control vs. 0.3 µM JNJ	Yes	*	0.0347
Row 25			
Control vs. 0.1 µM JNJ	No	ns	0.5402
Control vs. 0.3 µM JNJ	Yes	*	0.0142
Row 26			
Control vs. 0.1 µM JNJ	No	ns	0.664
Control vs. 0.3 µM JNJ	Yes	*	0.0133
Row 27			
Control vs. 0.1 µM JNJ	No	ns	0.6725
Control vs. 0.3 µM JNJ	Yes	*	0.0223
Row 28			
Control vs. 0.1 µM JNJ	No	ns	0.7591
Control vs. 0.3 µM JNJ	Yes	*	0.0389
Row 29			
Control vs. 0.1 µM JNJ	No	ns	0.8138
Control vs. 0.3 µM JNJ	Yes	*	0.0291
Row 30			
Control vs. 0.1 µM JNJ	No	ns	0.9576
Control vs. 0.3 µM JNJ	Yes	*	0.0272
Row 31			
Control vs. 0.1 µM JNJ	No	ns	0.8216
Control vs. 0.3 µM JNJ	Yes	*	0.047

7.1 IL-1R1 and P2X7R localization in hippocampus of murine brain.

Constitutive IL-1R1 expression was reviewed in saline treated CD-1 mice hippocampus. To understand which cell type expresses IL-1R1, brain tissues were counterstained with NeuN (neuronal marker).

Next, constitutive P2X7R expression was reviewed in saline treated CD-1 mice hippocampus. To understand which cell type expresses P2X7R in mice hippocampus, brain tissues were counterstained with NeuN (neuronal marker) or with P2Y12R (microglial marker).

7.1.1 Methods

Immunohistochemistry: as described in section 3.3.4 of chapter 3.

Antibody used:

Primary: **IL-1R1**, **NeuN, P2X7R, P2Y12R.**

Secondary: Goat anti-Mouse Alexa Flour 350, Goat Anti-Rabbit Alexa Fluor 488, Goat Anti-Rat Dylight 549, Goat anti-Hamster Dylight 405 (details in table 1.3)

7.1.2 Comments

IL-1R1 immunoreactivity is seen in granule cells of DG and also expressed in CA1 pyramidal neurons. Few CA3 pyramidal neurons also express IL-1R1 immunoreactivity. Endogenous P2X7R immunoreactivity is observed in both neuronal and non-neuronal cells of hippocampus.

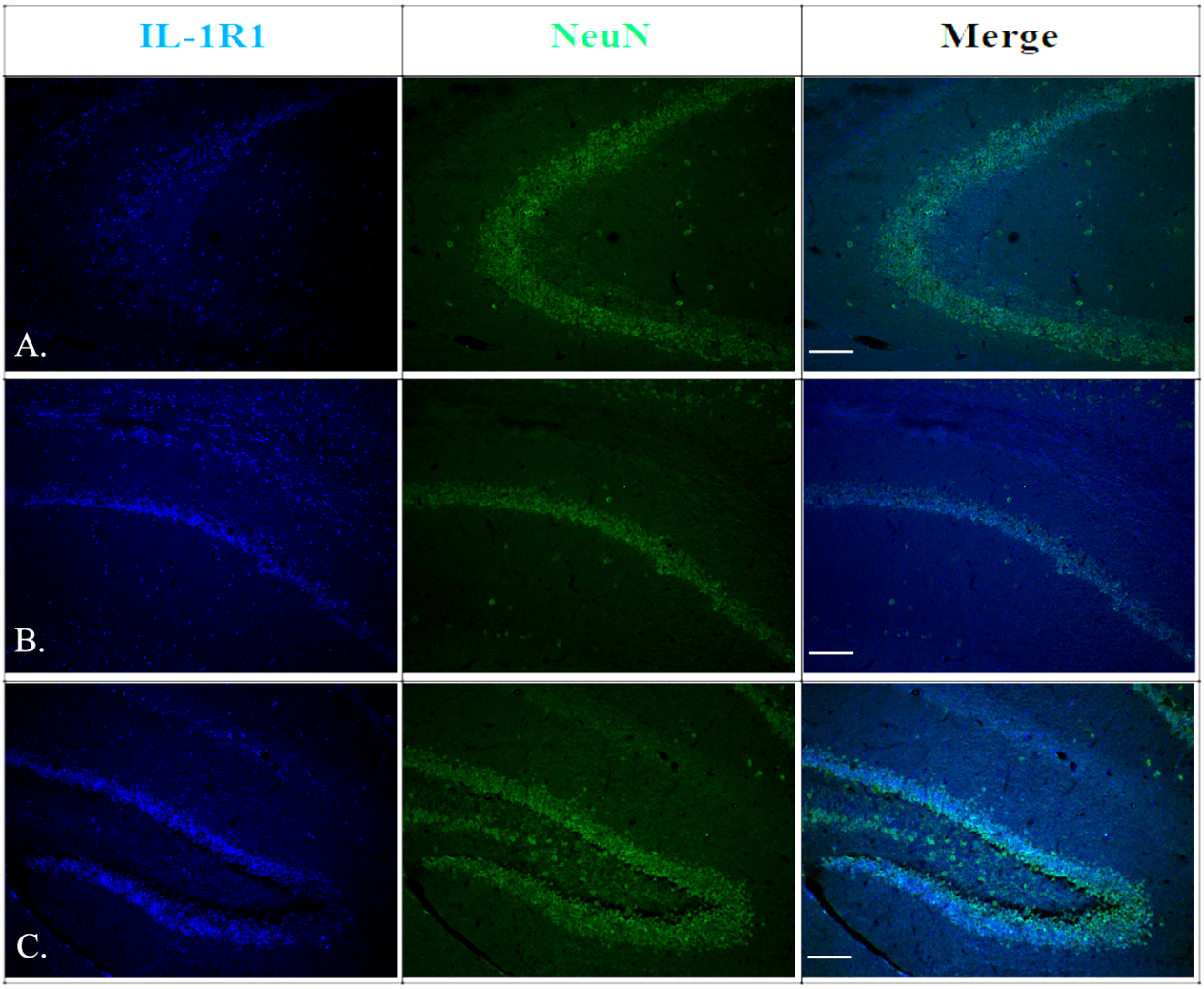


Fig 7.1. Localization of endogenous IL-1R1 immunoreactivity in neurons of the mice hippocampus.

Representative photomicrographs from saline treated mice brain section (panel **A.** CA3, **B**. CA1 and **C.** DG sub-regions of the hippocampus) demonstrated IL-1R1 immunoreactivity (blue) and NeuN (green) and merged (cyan) (10X objective). Headings indicate immunoreactivity coloration for each column of images. Scale bar = 100μM.

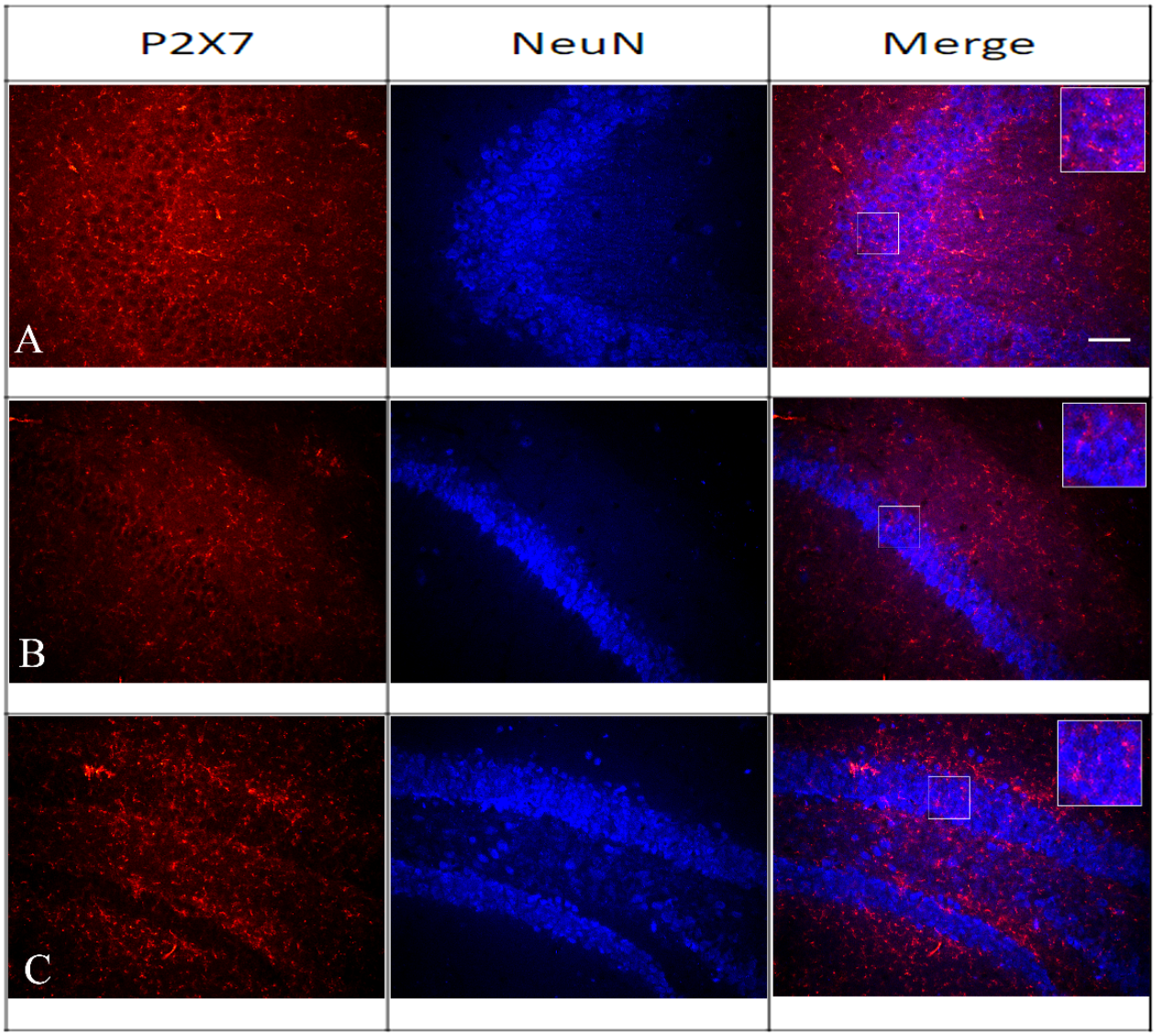


Fig 7.2. Constitutive P2X7R expression in neurons of hippocampus.

Representative photomicrographs from saline treated mice brain section (panel **A.** CA3, **B.** CA1, and **C.** DG sub-region of hippocampus) demonstrated P2X7 immunoreactivity (red), NeuN (blue) and merged (pink). (20X objective). Headings indicate immunoreactivity coloration for each column of images. Scale bar = 100μM. Inset in the merged section was enlarged to demonstrate P2X7 immunoreactivity in hippocampal neurons.

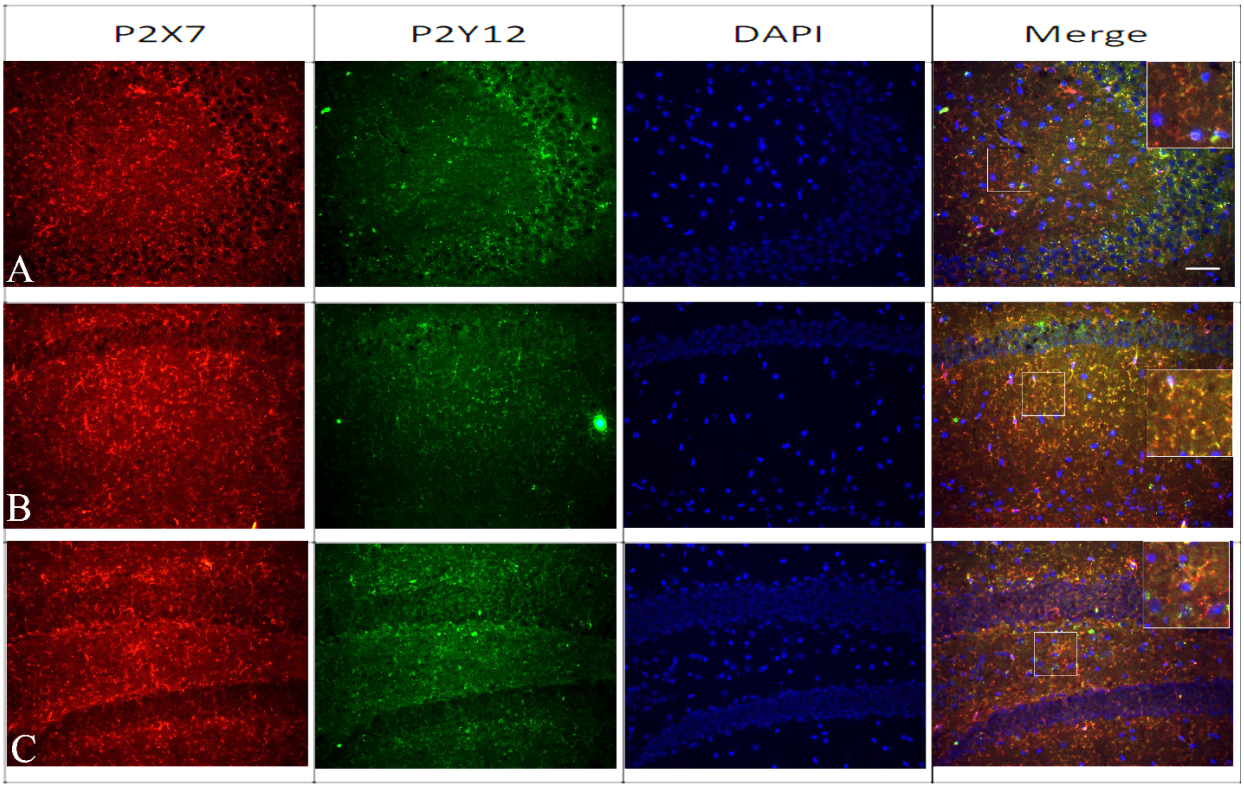


Fig 7.3. Constitutive P2X7R expression in non-neuronal cells of hippocampus.

Representative photomicrographs from saline treated mice brain section (panel **A.** CA3, **B.** CA1, and **C.** DG sub-region of hippocampus) demonstrated P2X7 immunoreactivity (red), P2Y12 (green), DAPI (blue) and merged (20X objective). Headings indicate immunoreactivity for each column of images. Scale bar = 100µM. Inset in the merged section was enlarged to demonstrate P2X7R immunoreactivity in microglial cells in mice hippocampus.

7.2 IL-1β Antibody validation

IL-1β antibodies (Ab) used for studying expression (ICC and WB) were validated prior to utilization.

7.2.1 Methods

Western blot analysis: as described in section 3.3.8 of chapter 3.

Immunocytochemistry: as described in section 3.3.5 of chapter 3.

Fluorescence intensity quantification: as described in section 4.3.7.4 of chapter 4.

7.2.2 Comments

IL-1β antibodies, i) rabbit anti-IL1β polyclonal antibody, 1:500 (Abcam Cat# ab9722, RRID:AB_308765) and ii) Rabbit polyclonal anti-IL-1β IgG, 1:100 (H-153, 200 µg/mL, Santa Cruz Biotechnology, Cat# sc-7884, RRID:AB_2124476) were validated with western blot using the recombinant mouse IL-1β peptide (Peprotech Inc., Cat# 211-11B) where protein expression was observed on the expected size (17 kDa for active IL-1β peptides and also on 31kDa, possibly pro-IL-1β peptide). (Fig 7.4)

Rabbit polyclonal anti-IL-1β IgG, 1:250 (H-153, 200 µg/mL, Santa Cruz Biotechnology, Cat# sc-7884, RRID:AB_2124476) was also validated through lowering of its immunoreactivity level when pretreated with recombinant mouse IL-1β blocking peptide (the antibody was incubated with 4X blocking peptide and mixed by gentle rocking on a shaker for 30 min in room temperature). Hippocampal neurons were treated either with DMSO (control) or with 0.3µM JNJ and then immunostained with either only IL-1β Ab or IL-1β Ab + blocking peptide. IL-1β expression significantly elevated with JNJ treatment. Blocking peptide bound antibody showed

reduced fluorescence intensity in both control and JNJ treatment, indicating specific binding of the antibody in question to IL-1β protein (Fig 7.5).

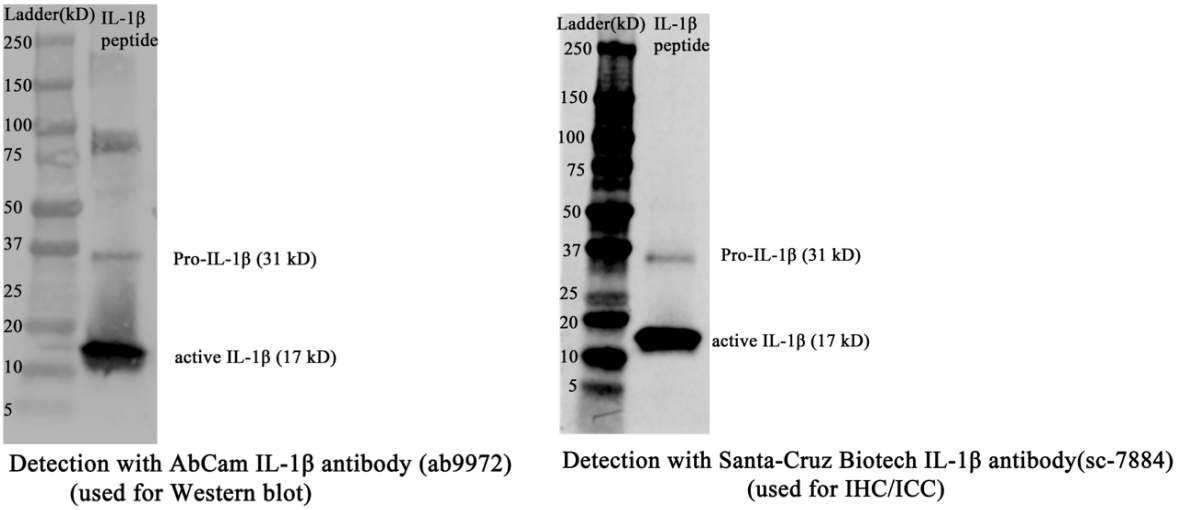


Fig.7.4. Immunoblots to verify the specificity of antibodies which bind to IL-1β peptide.

Blots showing antibodies' immunoreactivity to IL-1β (Abcam Cat# ab9722, RRID:AB_308765 on left and H-153, 200 µg/mL, Santa Cruz Biotechnology, Cat# sc-7884, RRID:AB_2124476 on right) by detection of the active IL-1β and possibly pro-IL-1β band in recombinant mouse IL-1β peptide. The known molecular weight is indicated through protein molecular weight ladder that was ran parallelly.

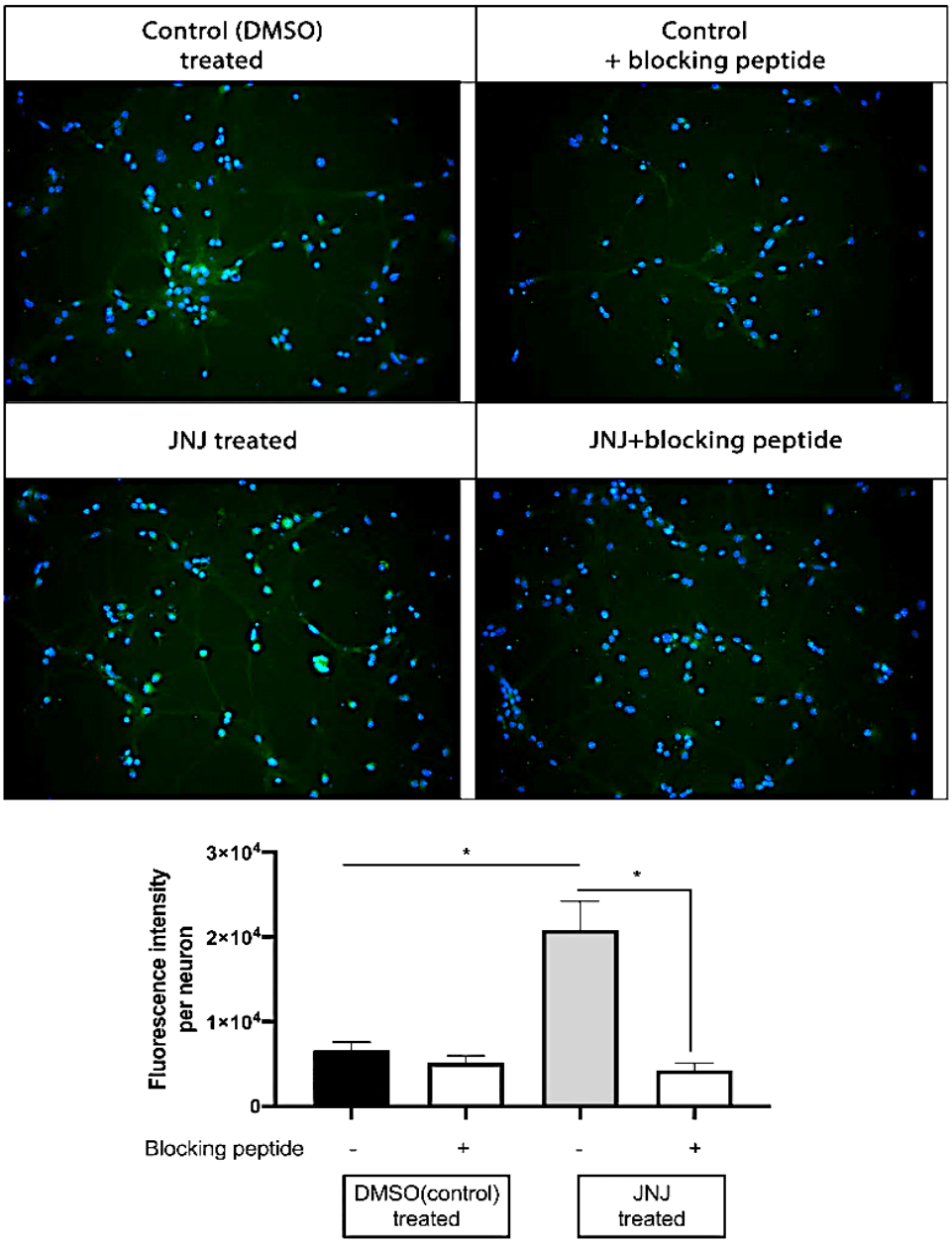


Fig.7.5. IL-1β immunoreactivity with and without pretreatment of blocking peptide: antibody validation.

Representative photomicrograph of hippocampal neurons in culture (DIV 14) showed constitutive expression of IL-1β (green) co-stained with DAPI (20X objective). Headings indicate specific treatment for individual image. IL-1β fluorescence intensity between treatments were compared by ordinary one-way ANOVA test followed by uncorrected Fisher's LSD test for multiple comparison (*,$p<0.001$).

7.3. Sub-cellular localization of IL-1β in hippocampal neurons may be extra-synaptic.

High-resolution microscopy was utilized to further understand if the subcellular localization of IL-1β is synaptic in hippocampal neurons *in vitro* and to identify if P2X7R antagonist caused any change in the compartmentalization of IL-1β immunoreactivity within these cells. For this study, hippocampal neurons were counterstained with synaptic terminal marker, Synaptophysin, and post-synaptic density marker, PSD-95 and compared between DMSO and JNJ treated neurons.

7.3.1 Methods

Immunocytochemistry: as described in section 3.3.5 of chapter 3.

Colocalization analysis: Co-localization tests are readily available in FIJI to estimate Meander's Coefficient and Pearson's coefficient via plugins. Setting correct threshold for estimating co-localization using these plugins is important as it may generate false-positive results unless done rightly. Prior to setting threshold for colocalization test, images (merged z-stacks) were checked for any colocalization via orthogonal view function through its z-axis utilizing FIJI. Only in case of images that provided any color merge in orthogonal view (yellow in color merge of red and green/cyan in green and blue/magenta in blue and red), colocalization tests were run.

7.3.2 Comments

Fig. 7.6 demonstrated cells with IL-1β immunoreactivity counterstained with PSD-95 without (Fig..7.6, Panel A) and with JNJ treatment (Fig.7.6, Panel B). The XY coordinates showed no visible colocalization with PSD-95 (absence of yellow) across the z-axis (5μm). Similarly, IL-1β immunoreactivity did not co-localize with synaptophysin either with DMSO (Fig.7.7, Panel A) or with JNJ treatment (Fig.7.7, Panel B) as seen through absence of cyan coloration across the z-axis (5 μm). All the fields were tested similarly to identify if there was any colocalization to indicate IL-1β to have synaptic presence. Preliminary studies herein, indicated otherwise.

This study indicated absence of IL-1β immunoreactivity either in synaptic terminal or in post synaptic density as seen through absence of co-localization with Synaptophysin or PSD-95. This may suggest sub-cellular localization of IL-1β to be extra-synaptic. Alternatively, it may be still synaptic, but not in the similar subcellular domain which contains synaptophysin or PSD-95. However, further investigations using confocal microscopy and co-localization techniques will be required to confirm this fact.

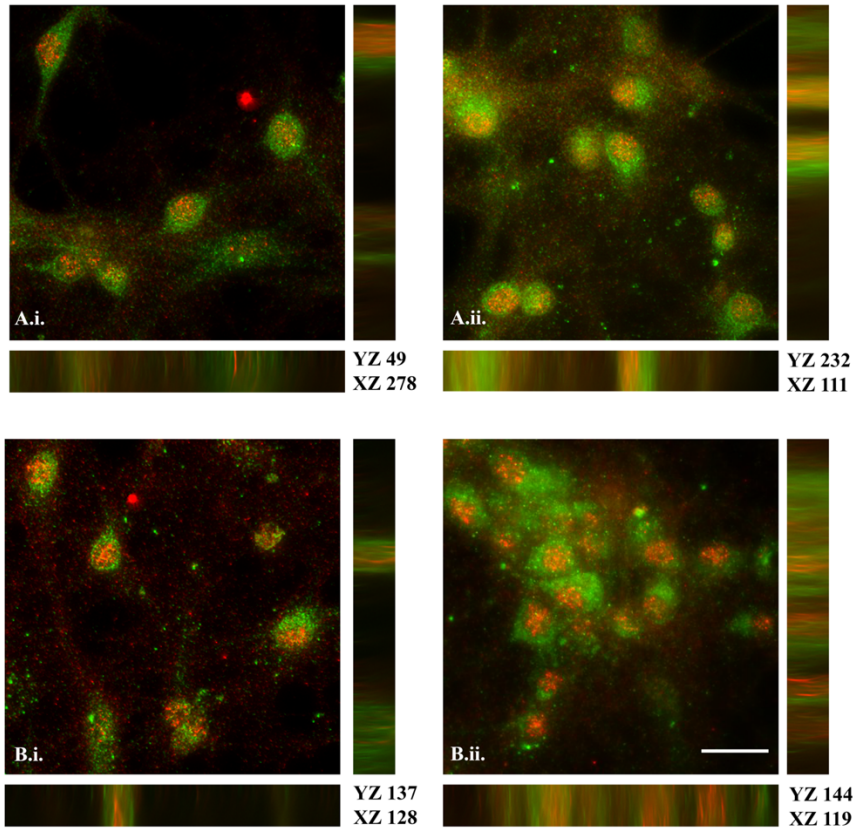


Fig.7.6. IL-1β may not be localized in the postsynaptic density.

Deconvolution images of **A.** DMSO and **B.** 0.3μM JNJ treated hippocampal neuronal cells in culture (DIV 14) demonstrated IL-1β (green) immunoreactivity co-stained with PSD-95 (red) to identify postsynaptic density. Each treatment is represented with two microscopic fields (**i** and **ii**) (40X objective). Figure panel on bottom and right of each image represents the orthogonal view [pixels of X and Y axis (enlarged) across z-axis (XZ and YZ pixel coordinates mentioned at bottom right)]. Scale bar = 2.5μm.

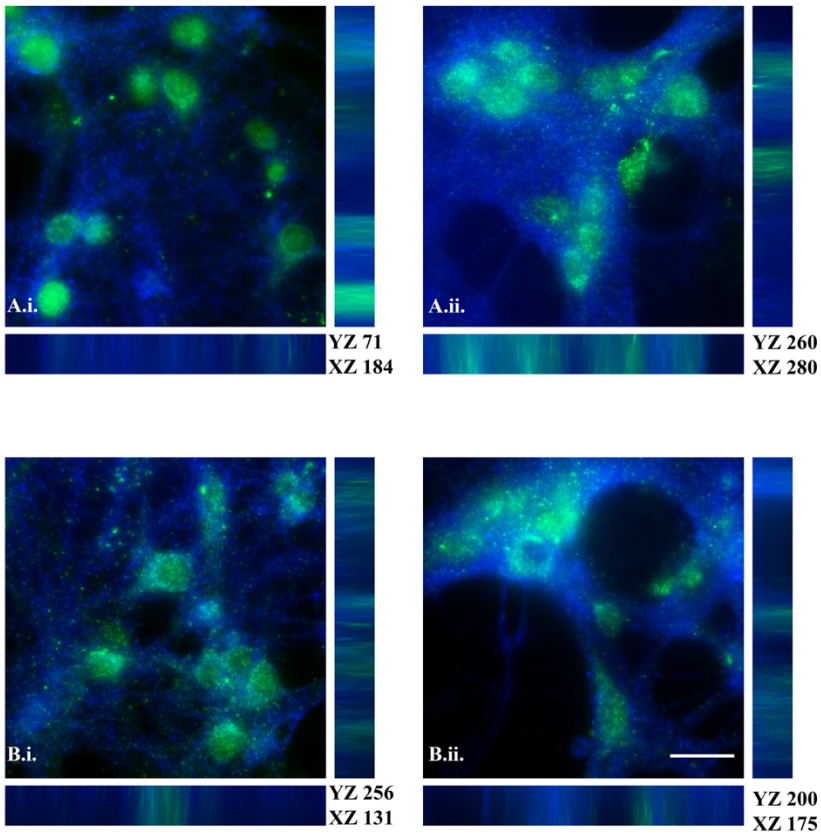


Fig.7.7. IL-1β may not be localized in the presynaptic terminal.

Deconvolution images of **A.** DMSO and **B.** 0.3μM JNJ treated hippocampal neuronal cells in culture (DIV 14) demonstrated IL-1β (green) immunoreactivity co-stained with synaptophysin (blue) to identify synaptic terminal. Each treatment is represented with two microscopic fields (**i** and **ii**) (40X objective). Figure panel on bottom and right of each image represents the orthogonal view [pixels of X and Y axis (enlarged) across z-axis (XZ and YZ pixel coordinates mentioned at bottom right)]. Scale bar = 2.5μm.

7.4 Effect of Caspase-1 inhibition on IL-1β release from hippocampal neurons *in vitro.*

7.4.1 Methods

Ac-YVAD-CHO treatment**:** To inhibit caspase-1mediated proteolytic cleavage of pro-IL-1β (31kD) to functional IL-1β (17kD) in hippocampal neurons, a stock solution of 50mM of Ac-YVAD-CHO was prepared in DMSO and stored at -20°C. This solution was diluted in maintenance medium and administered to DIV 14/15 cells for 60 min (for protein analysis) at concentrations of 10 or 30μM at 37°C in a humidified 5.5% CO2-containing-normotoxic incubator.

Western blot analysis: as described in section 3.3.8 of chapter 3.

7.4.2 Results

To understand the mechanism of activation of constitutive IL-1β prior to release, the cell-permeable Caspase-1 inhibitor, Ac-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO) (10 and 30μM) were added to the neuronal culture for 60 min and compared with vehicle-treated cultures. IL-1β expression in cell lysate was altered by Caspase-1 inhibition.

Caspase-1 inhibition treatment showed trends in increased expression of IL-1β in hippocampal neurons with 60 min treatment of 30μM yVAD-CHO as assessed by western blot analysis (Fig. 7.8). Western blot analysis showed the expression of only pro-IL-1β (31kD).

7.4.3 Comments

Caspase-1 inhibition treatment which exhibited increase (although not significant) in IL-1β in hippocampal neurons with 60 min treatment of 30μM yVAD-CHO may indicate role of Caspase-1 in IL-1β processing during release.

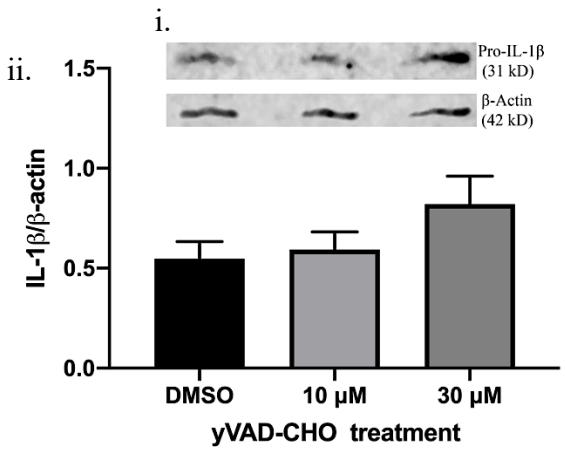


Fig. 7.8. IL-1β protein accumulated in hippocampal neurons with Caspase-1 inhibitor treatment.

Immunoblot analysis using anti-IL-1β and anti-β-actin antibodies was performed on cell lysates harvested after 1 hours following treatment with vehicle (DMSO) or yVAD-CHO (10μM and 30μM)as described in methods.

i. Representative blot showing IL-1β immunoreactivity compared to β-actin.

ii. Fluorescence intensity of IL-1β in cell lysate treated either with 10μM or 30μM yVAD-CHO (N=3,each treatment) or its vehicle (DMSO) (N=3) for 1 hour was quantified, normalized to β-actin fluorescence intensity and was analysed using ordinary one-way ANOVA (p=0.2397) followed by uncorrected Fisher's LSD test for multiple comparisons (p = 0.7705, DMSO vs. 10μM and p = 0.124, DMSO vs. 30μM yVAD-CHO). Equal variance was validated by Brown Forsythe test (p=0.1058, F (DFn, DFd) = (2, 6)).

7.5 Excitatory neuronal activity phosphorylates IRAK-1 in IL-1β dependent manner.

Interleukin-1 receptor-associated kinases (IRAKs) are serine threonine kinases for the interleukin-1 (IL-1) and for other members of the IL-1 receptor (IL-1R) and Toll-like receptor (TLR) family. Four IRAK molecules have been identified: two active kinases, IRAK-1 and IRAK-4, and two inactive, IRAK-2 and IRAK-M. IRAKs mediate activation of NF- kB and MAPK pathways. IRAK-4 functions upstream of IRAK-1 and phosphorylates it which then binds to TRAF-6 to mediate MAP kinase and NF-kB signaling (Li et al. 2002).In brain injury, IL-1R1 mediated IRAK-1/4 inhibition provided neuroprotection with rat brain injury model (Yang et al. 2011). Overexpression of Irak1 mimicked the reduced dendritic complexity of Mecp2-null cortical callosal projection neurons associated with Rett Syndrome (Kishi et al. 2016). These studies indicate role of IRAKs in neuroinflammatory diseases and neurological disorders. As referred in chapter 4, the goal of the study was to identify any changes in any downstream molecules of IL-1β signaling in acute seizure paradigm with an aim to identify a biomarker/effector molecule of IL-1β signaling in neuronal hyperexcitation. Phosphorylation of IRAK-1 was studied as one such candidate in PTZ induced convulsive seizure.

7.5.1 Methods

Mice

CD-1 mice: Brain sections of mice utilized for studies in chapter 4.

IL-1R1 mutant mice line: Brain sections of mice utilized for studies in chapter 2 & 4.

PTZ seizure paradigm

CD-1 mice: Acute seizure paradigm as described in methods () of chapter 3, PTZ dose of 60mg/kg was used.

IL-1R1 mutant line: Acute seizure paradigm as described in methods () of chapter 4, PTZ dose of 43.5mg/kg was used.

Immunohistochemistry: As described in section 3.3.3 (Chapter 3).

Antibody utilized: Primary – **pIRAK-1**, Secondary – Donkey anti-Rabbit Alexa Fluor 488

Immunofluorescence quantification - as described in section 3.3.3.4 (Chapter 3).

7.5.2 Results

7.5.2.1 Change in the phosphorylation level of IRAK-1 with acute convulsive seizure may be dependent on IL-1β signaling.

Time course (15 min, 30 min and 60 min following PTZ injection in CD-1 mice) study showed a significant transient increase in phosphorylated IRAK-1 immunoreactivity in all three sub-regions of hippocampus within 15 min of PTZ injection. Although, there was no p-IRAK-1 immunoreactivity in saline-treated mice hippocampus, mice with PTZ induced convulsive seizure showed an immediate bi-phasic increase in p-IRAK-1(lowered expression at 30 min and goes up again by 1 hour). The phosphorylated protein immunoreactivity was seen to be significantly elevated even at 1 hour following PTZ injection (Fig.7.9i) as quantified in Fig.7.9ii. To test if IRAK-1 phosphorylation was dependent on IL-1β signaling, both saline and PTZ treated wildtype and IL-1r1 mutant mice brain section were stained for p-IRAK-1 immunoreactivity. Similar to saline treated CD-1 mice, no p-IRAK-1 immunoreactivity was found in saline treated hippocampi of C57BL/6J mice (both wildtype and IL-1r1 mutant littermates) (Fig. 7.10). However, p-IRAK-1 immunoreactivity was significantly lower in all three sub-regions of hippocampus in KO mice compared to the WT littermates 1 hour after PTZ induced convulsive seizure (Fig. 7.10 i & ii). This study indicated IRAK-1 phosphorylation in mice due to excitatory neuronal activity may be dependent on IL-1β signaling.

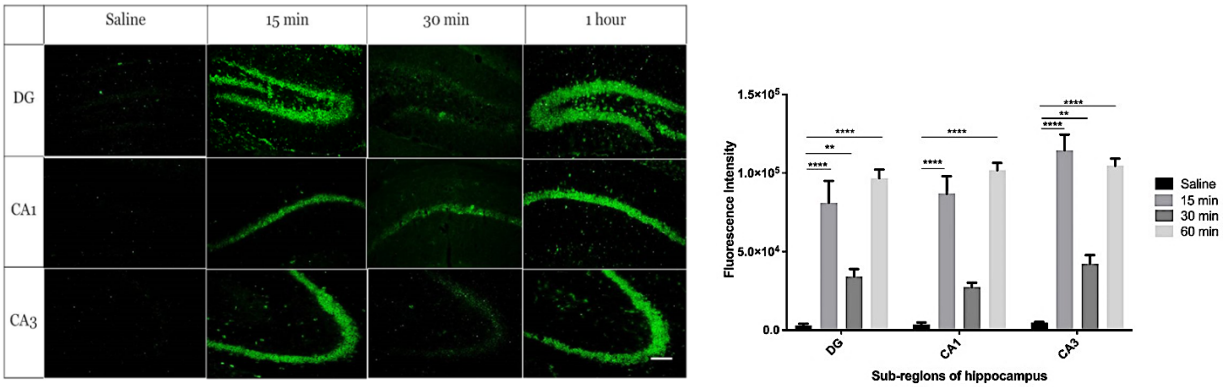


Fig.7.9. p-IRAK1 immunoreactivity in mice hippocampus following acute convulsive seizure.

i. Representative photomicrograph of mice hippocampus with p-IRAK1 (green) immunoreactivity following PTZ induced convulsive seizures in mice [60mg/kg b.w., 15 min, 30 min and 60 min (N=3,each time point)] and saline-treated mice hippocampus (N=3) (10X objective). Headings indicate time-points for each column of images. Scale bar =100µm.

ii. Immunofluorescence intensity in the subregions of hippocampus was quantitated as described in Materials and Methods and results are the average from duplicate sections. Significant differences were analyzed using 2-way ANOVA followed by Dunnett's multiple comparisons test. (p-IRAK1 immunoreactivity post injection, a, significantly different from respective vehicle-treated controls (*, $p<0.001$) and b, significantly different between subregions (*, $p= 0.024$)). No p-IRAK1 expression were detected in the saline treated mice.

Dunnett's multiple comparisons test	Significant?	Summary	Adjusted P Value
DG			
Saline vs. 15 min	Yes	****	<0.0001
Saline vs. 30 min	Yes	**	0.0092
Saline vs. 60 min	Yes	****	<0.0001
CA1			
Saline vs. 15 min	Yes	****	<0.0001
Saline vs. 30 min	No	ns	0.0531
Saline vs. 60 min	Yes	****	<0.0001
CA3			
Saline vs. 15 min	Yes	****	<0.0001
Saline vs. 30 min	Yes	**	0.0019
Saline vs. 60 min	Yes	****	<0.0001

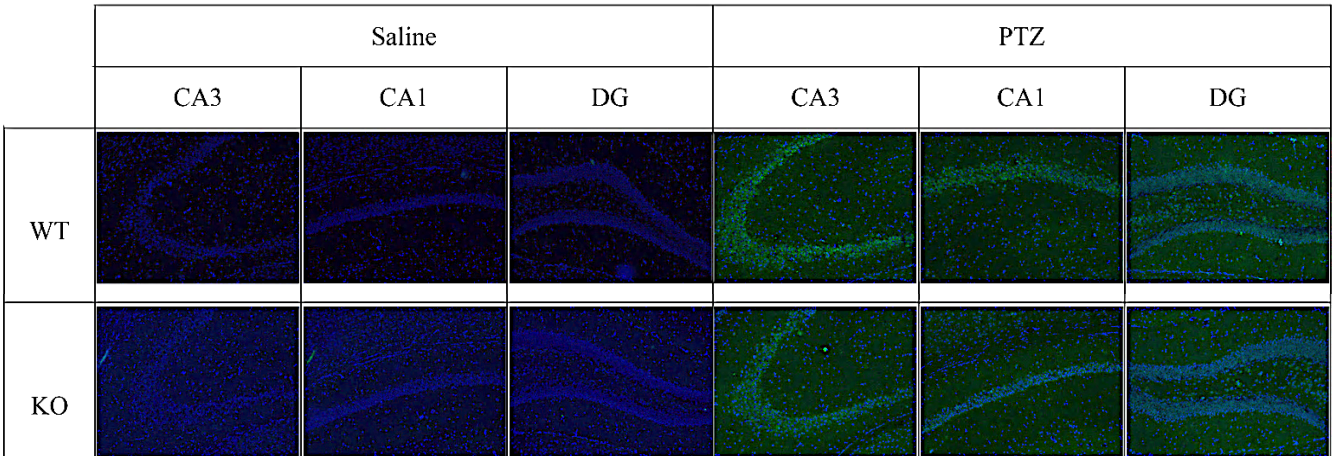
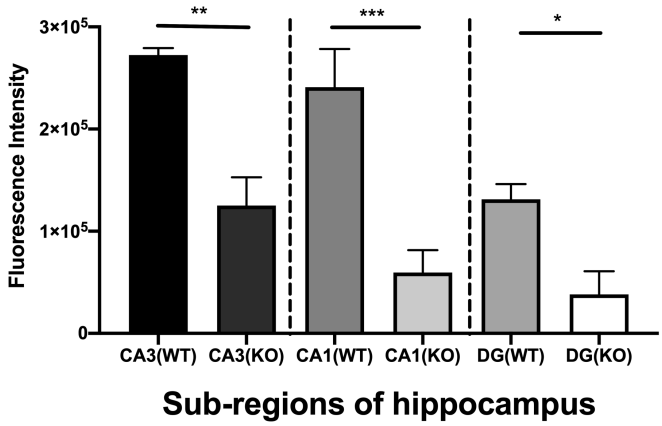


Fig.7.10 IRAK-1 phosphorylation may be dependent on IL-1β signaling.

i. Representative photomicrograph of WT and IL-1R1 KO mice hippocampus with p-IRAK1 (green) immunoreactivity with saline or PTZ treatment [60mg/kg b.w., 60 min post injection (N=4,each time point)] (10X objective). Headings indicate sub-regions of hippocampus under specific treatment for each column of images. Scale bar =100µm.



i. Immunofluorescence intensity in the subregions of hippocampus of WT or IL-1R1 KO mice 1hour post PTZ injection was quantitated as described in Materials and Methods and results are the average from duplicate sections. Significant differences were analyzed using 2-way ANOVA followed by Bonferroni's multiple comparisons test. [p-IRAK1 immunoreactivity post injection, a, significantly different within genotypes (*, $p<0.001$) and b, significantly different between subregions of hippocampus (*, $p= 0.006$)].

Bonferroni's multiple comparisons test	Significant?	Summary	Adjusted P Value
WT - KO			
CA3	Yes	**	0.0011
CA1	Yes	***	0.0001
DG	Yes	*	0.0396

7.6 To test the possibility that IL-1 signaling in the DG of hippocampus is key to the maintenance of the innate seizure threshold.

7.6.1 Introduction

As mentioned in Aim 1, mice lacking the obligate signaling receptor for IL-1 signaling, IL-1R1, are more prone to convulsive seizures (i.e., have a reduced seizure threshold). Basal expression of the IL-1R1 is particularly prominent in the neurons of the dentate gyrus (DG), with lower expression in the CA3-CA4 regions (Parnet et al. 1994; Eriksson et al. 1999; Friedman 2001). Consistent with this, ligand binding studies indicate that the IL-1 binding is highly concentrated in the DG (Ban et al. 1991). Together, these results raise the intriguing possibility that the neuromodulatory effects of IL-1β may be concentrated in the DG. The hippocampal formation plays an important role in the process of seizure induction and epileptogenesis (Sloviter 1994; Sutula et al. 1998; Ang, Carlson, and Coulter 2006; Fujita et al. 2014; Sloviter 2005). The DG is an important control point for information entering the hippocampus from the entorhinal cortex particularly in the epileptic brain (Gloveli, Schmitz, and Heinemann 1998; Sutula and Dudek 2007; Houser et al. 2012; Ang, Carlson, and Coulter 2006; Kobayashi and Buckmaster 2003). Because IL-1β is released in the hippocampus and its binding sites are concentrated in the granular cell layer of the DG (Ban et al. 1991; French et al. 1999; Friedman 2001), the DG may be a site of IL-1β signaling and loss of signaling in the DG may account for the seizure phenotype of IL-1RI mutant mice.

These studies will also be pertinent because 1) the DG is considered a key regulatory point in the circuitry between the cortex and hippocampus and 2) this circuit is implicated in epilepsy, the clinical relevance of this proposal (Sloviter 1994; Sutula et al. 1998; Sloviter 2005; Ang, Carlson, and Coulter 2006). Using a novel Cre-dependent transgenic approach that permits cell-

type specific restoration of functional IL-1 signaling in the null background which will permit to restore IL-1 signaling in the DG areas of the hippocampal formation in IL-1R1 deficient mice. If IL-1 signaling in these neuronal population is necessary to maintain the innate seizure threshold the goal is to restore IL-1 function will re-establish the wild-type phenotype (Aim 1).

7.6.2 Methods

7.6.2.1 Mice:

7.6.2.1.1 POMC-Cre colony: Cre-dependent DNA recombinase to DG granule cells using the proopiomelanocortin (POMC) gene promoter [POMC-Cre;(McHugh et al. 2007)] in mouse targets recombination in the DG specifically. This promoter is transcriptionally activated during the late postnatal period of development, lessening the possibility of developmental effect of the transgenic manipulation. Importantly, our laboratory has shown that the cross between this line and a line harboring a loxP-flanked transgene is capable of driving recombination of a transgene in the hippocampal formation of mice but not in the cortex. This mouse line, which is referred to as POMC-Cre (Cre/+), is available from Jackson Laboratory (stock no. 010714).

Breeding: The Cre hemizygous males were crossed with C57BL/6J WT female mice for maintenance of the line. Due to leakiness of Cre gene reported, for Cre-Lox breeding, it is advisable to maintain this Cre in males.

Genotyping: Genotyping was done following section 2.3.2 on Chapter 2. All genotyping was performed through PCR analysis (Protocol 22392, The Jackson Laboratory) of tail genomic DNA samples using allele-specific primers ((50μM), Integrated DNA technologies):

Cre1a: 5’- GTA ACT AAA CTG GTC GAG CGA TG-3’

Cre1b: 5’- CTG TTT CAC TAT CCA GGT TAC GG -3’

7.6.2.1.2 IL-1R1 restore colony: This mouse line on C57BL/6N harbors a functional IL1RI transgene that is transcriptionally activated by Cre in the background of global IL1RI gene deficiency (X. Liu et al. 2015). The transgenic construct was inserted in the endogenous IL-1RI gene, thus inactivating it. Once the transgene is activated by Cre, transcription of a function receptor is driven by the endogenous IL-1RI gene promoter region. Transgene expression can be monitored at the mRNA or protein level using tdTomato fluorescence and 3xHA epitope, respectively. This mouse line, which is referred to as IL-1RI$^{r/r}$, is available from Jackson Laboratory (stock no. 024101).

Breeding: Filial male wildtype (WT or +/+) and mutant (IL-1R1 restore or r/r) littermates for studies were derived from parental heterozygous (Het or +/r) breeding units that were obtained from Jackson Laboratory after recovering the cryogenically preserved embryos. The female and the male hets (+/r) were utilized for subsequent breeding. The above breeding and caging strategy followed previous study (Claycomb, Hewett, and Hewett 2012) to control for potential non-specific differences in environmental or genetic factors.

Genotyping: Genotyping was done following section 2.3.2 on Chapter 2. All genotyping was performed through PCR analysis (Protocol 28334, The Jackson Laboratory) of tail genomic DNA samples using allele-specific primers ((50μM), Integrated DNA technologies):

PRIMER	SEQUENCE 5' → 3'	PRIMER TYPE
19775	TGC ATC GCA TTG TCT GAG TAG	
23160	TCA GAG ATG AAA TGA CTA CAA GCT G	Common
23550	TTG TGC ATA AAG TGA ATG CTA GTG	Wild type Forward

7.6.2.1.3 Cre-Lox breeding scheme: To restore the IL-1R1 expression in the DG, the POMC-Cre^{+} and IL1RI$^{r/r}$ lines (Fig.7.11) were crossed. An initial cross between female IL1R1$^{r/r}$ (IL1R1$^{-/-}$) and male POMC-Cre^{+} (IL1R1$^{+/+}$) mice would yield POMC-Cre^{+}/ IL1R1$^{+/r}$ offspring. These males are back-crossed with female IL-1R1$^{r/r}$ to yield POMC-Cre^{+}/ IL-1R1$^{r/r}$ offspring.

These mice will be IL-1R1$^{+/+}$ in the DG granule cells (IL1R1$^{DG/DG}$/POMC-Cre^{+}), while all other cell types will be IL1R1$^{-/-}$.

IL-1RI$^{r/r}$ X POMC-Cre^{+} (IL-1RI$^{+/+}$)	Parental Cross
IL-1RI$^{+/r}$/POMC-Cre^{+} X IL-1RI$^{r/r}$	Back Cross
IL-1RI$^{DG/DG}$/POMC-Cre^{+}	IL-1RI restored in DG

Fig.7.11 Breeding scheme for the study.

7.6.2.2 PTZ seizure paradigm: described in section 2.3.3 of chapter 2.

7.6.2.2.1 Dose response study: Male wildtype C57BL/6N and IL-1R1$^{r/r}$ were dosed with 43.5mg/kg PTZ as this dose showed an array of seizure response in IL-1R1 traditional KO colony (Fig 2.2A).

For this analysis, littermates of wild-type (WT) C57BL/6N$^{+/+}$ andIL-1R1 restore (IL1R1-deficient globally) genotypes from breeding heterozygous IL-1RI $^{r/+}$ were dosed with 43.5 mg/kg and seizure behavior was scored as described. Although a greater number of KO showed more severe seizure, the difference was not significant. The restore mice and WT mice which showed the median score of 4 (p= 0.2, Mann Whitney Test, two tailed) (Fig. 7.12 A). To represent incidence of convulsion, although there is a trend of higher incidence of convulsion in restore mice, Fisher's exact test showed no significant difference in restore mice (100%) having convulsive seizures compared to WT littermates (67%) (p= 0.4667, 2x2, two tailed Fisher's exact test) (Fig. 7.12 B).

Although the result did not yield any significant difference between the genotypes, it generated a trend similar to IL-1R1 traditional KO colony. One of the reasons may be that, as PTZ induced

seizure occurs in a dose dependent manner, this dose of 43.5 mg/kg is approaching a ceiling effect and a lower dose should be tested to see the array of seizure response.

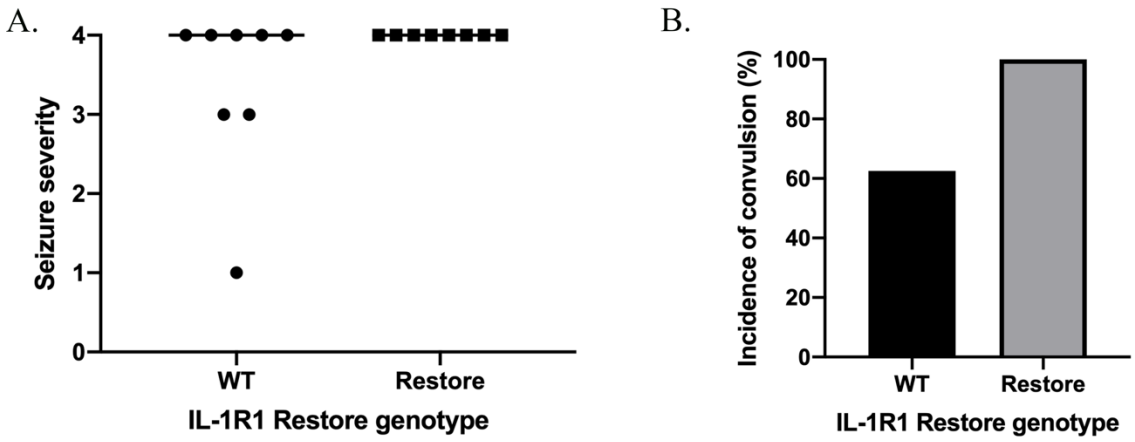


Fig.7.12 The innate seizure threshold of mice lacking IL-1 signaling.

Mice from each genotype (WT (*N*=8) and Restore(*N*=8)) were treated with 43.5mg/kg b.w. PTZ and seizure behavior were scored a 5-point scale of increasing severity as described in the methods.

A. Seizure severity. Each point represents the maximum seizure score for an individual mouse. The median seizure scores within the two genotypes were statistically significant (p= 0.2, Mann Whitney Test, two tailed).

B. Incidence of convulsions. Histogram represents the % convulsing to total number of mice injected with PTZ.

7.6.3 Cre-Lox Breeding

Crossing POMC-Cre with IL-1R1 restore mice yielded $POMC^{Cre/+}$/$IL\text{-}1R1^{r/+}$ and $POMC^{+/+}$/$IL\text{-}1R1^{r/+}$ genotypes. However, owing to leakiness of Cre in females only $POMC^{Cre/+}$/$IL\text{-}1R1^{r/+}$ males were backcrossed with IL-1R1r/r females yielding 4 different genotypes, $POMC^{Cre/+}$/$IL\text{-}1R1^{r/+}$ (the IL-1R1 gene will be functional in DG of hippocampus in brains of this genotype in heterozygous fashion), $POMC^{+/+}$/$IL\text{-}1R1^{r/+}$, $POMC^{+/+}$/$IL\text{-}1R1^{r/r}$ and $POMC^{Cre/+}$/$IL\text{-}1R1^{r/r}$ (the IL-1R1 gene will be functional in DG of hippocampus in brains of this genotype). Three cohorts of breeding utilizing breeding pairs yielded a total of 34 mice.

When genotyped, I obtained,

$POMC^{+/+}$/IL-1R1$^{r/+}$	$POMC^{+/+}$/IL-1R1$^{r/r}$	$POMC^{Cre/+}$/IL-1R1$^{r/+}$	$POMC^{Cre/+}$/IL-1R1$^{r/r}$
Males			
7	4	4	1
Females			
5	3	10	0

Table 7.2. Total offspring obtained from Cre-Lox breeding.

7.6.4 Unprecedented problems in breeding and maintenance of colony:

IL-1R1 restore line: This line was restored from cryogenic preservation of embryos. When maintained in our animal house, several mice of this colony irrespective of gender or genotypes suffered from mild to severe hair loss followed by ulcerative dermatitis in extreme cases particularly in winter. Several males and females (35%) which had mild to medium ulcerative dermatitis failed to mate or become pregnant reducing the total fecundity.A distinct number of hets and restore mice irrespective of age would develop constant shaking of unreported nature which developed around 10-12 weeks of age. As the Cre-Lox recombination occurred around 12-16 weeks, these mice were scheduled for the PTZ study around 13-16 weeks. However, mice that showed this symptom were taken out of the study. Several breeders (20%) were also euthanized and could not be used because of this condition, reducing the total fecundity and mice obtained through breeding.

POMC-Cre Hemizygous/IL-1R1 restore het males: The expected ratio of obtaining male double transgenic genotype is $1/8^{th}$ of total pups according to Mendelian law. However, in nature, such ratio varies widely sometimes lowering the actual number of the desired genotype.

The IL-1R1 restore/Cre hemizygous males showed an unique phenotype, which hindered our breeding process severely. Firstly, after following general rule of breeding and setting up younger females with older males to avoid aggressive behavior, several of the males were attacked in their genitalia by female IL-1R1$^{r/r}$ mice. In one breeding cohort, 3 out of 5 males were attacked severely, out of which 2 died due to excessive bleeding. The remaining one had to be euthanized as per veterinarian's advice. Out of the surviving males, out of 2 males from that cohort and 4 from the next, 3 died of unknown cause (as reported by Laboratory animal research personnel) at age of 14-16 weeks. Unprecedented deaths of these rare breeders affected the entire Cre-Lox breeding process in terms of cost and time required to breed a generation of littermates.

Obtaining double transgenics:

As seen in table 7.2, with 3 cohorts of breeding, I did not obtain any female double transgenic (POMC$^{Cre/+}$/IL-1R1$^{r/r}$ females) although the chances of obtaining this particular genotype was 1/8th or out of female mice. Similarly, I obtained only 1 male double transgenic (POMC$^{Cre/+}$/IL-1R1$^{r/r}$ males), although chances of obtaining this particular genotype was again 1/8th or out of male mice. Although, the number is too small to pinpoint towards any developmental reasons, but it may be indicative of either embryonic lethal or neonatal deaths of these double transgenic pups. As, the entire study was pivoted on the seizure phenotype of the double transgenic, not obtaining any through all the breeding stalled the entire study. In research, we often come across unprecedented problems or pitfalls, which required troubleshooting. As breeding requires resources and is time consuming, often troubleshooting may not provide the answer. We looked for alternative strategies. Instead, studying the tissue specific relevance of IL-1 signaling in mice utilizing IL-1R1conditional knock out mice which will selectively knock-out IL-1 signaling was proposed, which may be able to bypass these above-mentioned complications.

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PUBLICATIONS:

Soham Mitra, Nilkanta Chakrabarti, **Spandita S Dutta**, Sucharit Ray, Promita Bhattacharya, Priyobrata Sinha, Arindam Bhattacharyya. "Gender specific brain regional variation of neurons, endogenous oestrogen, neuroinflammation and glial cells during rotenone induced mouse model of Parkinson's disease."
DOI: https://doi.org/10.1016/j.neuroscience.2014.12.052.

Spandita S. Dutta, Torsten Wöllert, Sandra J. Hewett and James A. Hewett. P2X7-dependent constitutive Interleukin-1β release from pyramidal neurons of the mouse hippocampus: Role in maintenance of the innate seizure threshold (Manuscript submitted).

INVITED PRESENTATIONS:

Invited graduate student talk at **5th SU Neuroscience Day, Syracuse University**, **April 5th, 2019,** on P2X7R antagonism regulating neuronal excitation through inhibition of Interleukin-1β release both *in vivo* and *in vitro*.

POSTERS:

- **Spandita S. Dutta, Dr James A. Hewett**. Constitutive neuronal Interleukin -1β release: Influence on neuronal excitation. International and American Societies of Neurochemistry conference 2019, August 2019, Montreal, Canada.
- **Spandita S. Dutta, Dr James A. Hewett**. Poster title: P2X7 receptor antagonism lowers the innate seizure threshold possibly through inhibition of Interleukin-1β release. Society for Neuroscience conference, November 2017, Washington D.C.
- **Spandita S. Dutta, Dr James A. Hewett.** Poster title: "Interleukin-1β may function as a neuromodulatory pathway to restrain excessive excitatory neuronal activity." American Society of Neurochemistry conference, March 2016, Denver, Colorado.

HONORS & AWARDS:

- Future Professionals program, WISE-SU, 2015-2017
- Alpha Sigma chapter, PHI BETA DELTA, Honor Society for International Scholars (2015)

- International society for Neurochemistry meeting (Montreal, Canada) 2019 travel award
- Neuroscience Graduate Student Travel Award, Syracuse University

  - ISN-ASN conference, Montreal, Canada, August 2019
  - Society for Neuroscience meeting, Washington D.C., November 2017
- Dept of Biology & Graduate Student Organization, Syracuse University travel awards

  - ISN-ASN conference, Montreal, Canada, August 2019
  - Society for Neuroscience meeting, Washington D.C., November 2017
  - American Society for Neurochemistry, Denver, Colorado, March 2016

- Endowment Fellowship, University of Calcutta, Kolkata, India, 2013
- Junior Research Fellowship, Department of Science and Technology, India (Aug. 2012 - July 2012)
- Indira Gandhi Single Girl Scholarship, University Grants Commission, Govt. of India, India (Aug.2009-June 2011)

PROFESSIONAL ASSOCIATIONS:

- **Member,** Medical Science Liaison Association & CSA associate, Cheeky Scientists' Association, 2020-present
- **Member**, Society for Neuroscience, 2017 – Present
- **Member**, American Society for Neurochemistry, 2016 - Present
- **Member**, International Society for Neurochemistry, 2016 – Present
  Member, Women in Science and Engineering Future Professionals Program, SU Chapter, 2015 - Present
- **Member**, Alpha Sigma Chapter of Syracuse University, Phi Beta Delta International Honors Society, 2015- Present.